8th CONGRESS of the GENETICS SOCIETY of SLOVENIA

&

8th MEETING of the SLOVENIAN SOCIETY of HUMAN GENETICS

with INTERNATIONAL PARTICIPATION

GENETIKA 2018

BOOK OF ABSTRACTS

Matej Skočaj (Editor)

September 19–21, 2018

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Name badges

Name badges are required during all congress scientific sessions as well as during social events.

For speakers

Speakers are kindly requested to upload their presentation onto the computer in the lecture hall at least half an hour before the start of their respective session. Technical assistance will be provided.

For poster presenters

All posters will be displayed during the entire congress in the *Izvir* hall. Three best posters selected by an international jury will be awarded. The presence of the presenter at the poster is required during both poster sessions: on Thursday, 20th September, from 14:00 to 15:00, and on Friday, 21st September, from 11:00 to 12:00

Material for mounting the posters will be available at the venue. Presenters are responsible for setting and removing their own posters. Poster panels will be available from Wednesday, 8 a.m. to Friday, 5 p.m.

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CONGRESS PROGRAM

Wednesday, September 19

from 9:00 REGISTRATION

10:45 – 11:00 OPENING CEREMONY

Darja Žgur Bertok, Damjan Glavač

11:00 – 11:45 OPENING LECTURE

Chair: Darja Žgur Bertok

Borut Peterlin, University Medical Center, Ljubljana, Slovenia
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Co-chairs: Nadja Kokalj Vokač, Metka Ravnik-Glavač

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Balanced Chromosomal Rearrangements as Windows into the Developmental Regulome

12:10 – 12:30 **Špela Stangler Herodež**, University Medical Centre Maribor, Slovenia
Next Generation Sequencing (NGS) in Cardiology

12:30 – 12:45 Danijela Krgović, University Medical Centre Maribor and University of Maribor, Slovenia
Genomic Analysis in Childhood Schizophrenia

12:45 – 13:00 Emanuela Boštjančič, University of Ljubljana, Slovenia
Expression of Pro-Apoptotic miR-34a and its Target Genes in Human Myocardial Infarction and Healthy Heart Tissue

13:00 – 13:15 Matija Rijavec, University Clinic of Respiratory and Allergic Diseases Golnik, Slovenia
Hereditary Angioedema due to C1-inhibitor Deficiency: Heterogeneity of SERPING1 Mutations, and Genetic Factors Modifying the Clinical Phenotype

13:15 – 13:30 Uršula Prosenc Zmrzljak, Bia Separations CRO and Labena d.o.o, Slovenia
Detection of Somatic Mutations with ddPCR from Liquid Biopsy of CRC Patients

13:30 – 13:45 Mojca Milavec, National Institute of Biology, Slovenia
Quantification of Human Cytomegalovirus with Digital PCR

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Co-chairs: Nataša Štajner, Damjan Glavač

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18:45 – 19:00 Katja Repnik, University of Maribor, Slovenia
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Co-chairs: Darja Žgur Bertok, Damjan Glavač

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15:45 – 16:45	**The Genetics Society of Slovenia Annual Assembly**

16:45 – 17:00	**Best Posters and Golden Chromosome Award Presentation**

17:00 – 17:15	**Closing Ceremony**


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OPENING LECTURE

Borut Peterlin, University Medical Center, Clinical Institute of Medical Genetics (KIMG)

Genome medicine: from rare to complex disorders


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GENOME MEDICINE: FROM RARE TO COMPLEX DISORDERS

Borut Peterlin, University Medical Center Ljubljana, Clinical Institute of Medical Genetics (KIMG), Slovenia

Genomic medicine has revolutionized the approach to rare diseases. Implementation of next generation sequencing technology together with several technical and organizational innovations in the Slovenian health system significantly improved access to genetic testing, diagnostic and economic efficacy of testing and consequently much more patients receive the diagnosis of rare disease. Early etiological diagnosis shortens often long diagnostic odyssey and is necessary for potential specific or symptomatic treatment and prevention.
Moreover, systematic application of exome sequencing in the diagnosis of rare, undiagnosed conditions enabled us to discover new genes associated with human disorders including genes for the Fontaine syndrome, epileptic encephalopathy, microcephaly, cardiomyopathy and ciliopathy (1,2,3).
Finally, genomic screening tests provide opportunity for predictive, preventive, personalized and participatory medicine. Morbid genome atlas of the Slovene populations based on more than 2000 exomes analyzed so far at the KIMG, serves as an important information for designing the Slovene genome screening programmes, from the preconceptional, prenatal, neonatal and adult one.
While new genomic technologies already have an important impact for rare diseases, much more research is needed in the field of complex disorders. One of the disease models our group is investigating is multiple sclerosis, a debilitating neurological disease affecting young adults. Study of rare genetic variants with high penetrance brought us to identification and better understanding of monogenic factors involved in the pathogenesis of disease (4).

1. Tumienė B, Maver A, Writzl K, et al. (2018) Diagnostic exome sequencing of syndromic epilepsy patients in clinical practice. Clin Genet 93:1057-1062
2. Zaman T, Helbig I, Božović IB, et al. (2018) Mutations in SCN3A cause early infantile epileptic encephalopathy. Ann Neurol. 83:703-717
3. Writzl K, Maver A, Kovačič L, et al. (2017) De Novo Mutations in SLC25A24 Cause a Disorder Characterized by Early Aging, Bone Dysplasia, Characteristic Face, and Early Demise. Am J Hum Genet. 101:844-855
4. Maver A, Lavtar P, Ristić S, et al. (2017) Identification of rare genetic variation of NLRP1 gene in familial multiple sclerosis. Sci Rep. 7:3715


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ABSTRACTS OF LECTURES


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Niels Tommerup, Wilhelm Johannsen Centre for Functional Genome Research, Department of Cellular and Molecular Medicine (ICMM), University of Copenhagen, Copenhagen, Denmark

Balanced Chromosomal Rearrangements as Windows into the Developmental Regulome

Špela Stangler Herodež, Medical Clinical Centre Maribor, Laboratory of Medical Genetics, Slovenia

Next Generation Sequencing (NGS) in Cardiology

Danijela Krgović, University Medical Centre Maribor, Laboratory of Medical Genetics and University of Maribor, Medical Faculty, Department for Molecular Biology, Slovenia

Genomic Analysis in Childhood Schizophrenia

Emanuela Boštjančič, University of Ljubljana, Faculty of Medicine, Institute of Pathology, Department of Molecular Genetics, Slovenia

Expression of Pro-Apoptotic miR-34a and its Target Genes in Human Myocardial Infarction and Healthy Heart Tissue

Matija Rijavec, University Clinic of Respiratory and Allergic Diseases Golnik, Slovenia

Hereditary Angioedema due to C1-inhibitor Deficiency: Heterogeneity of *SERPING1* Mutations, and Genetic Factors Modifying the Clinical Phenotype

Uršula Prosenc Zmrzljak, Molecular Biology Laboratory, Bia Separations CRO, Labena d.o.o, Ljubljana, Slovenia

Detection of Somatic Mutations with ddPCR from Liquid Biopsy of CRC Patients

Mojca Milavec, National institute of biology, Department of Biotechnology and Systems Biology, Ljubljana, Slovenia

Quantification of Human Cytomegalovirus with Digital PCR

BALANCED CHROMOSOMAL REARRANGEMENTS AS WINDOWS INTO THE DEVELOPMENTAL REGULOME

Niels Tommerup[1], Mana M. Mehrjouy[1], Mads Bak[1], Peter Jacky[2], Nadja Kokalj Vokač[3], Andreja Zagorac[3], International Breakpoint Mapping Consortium[4]

[1]Wilhelm Johannsen Centre for Functional Genome Research, Department of Cellular and Molecular Medicine (ICMM), University of Copenhagen, Denmark
[2]NW Permanente, PC, Emeritus, Portland, Oregon, US
[3]Universty Medical Centre Maribor, Laboratory of Medical Genetics, Slovenia
[4]Participants from >100 cytogenetic laboratories world-wide

Balanced chromosomal rearrangements (BCRs) have been used for decades to identify disease loci, an approach which has been facilitated by global paired-end sequencing methods that can detect chromosomal breakpoints at near sequence level. BCRs do not only cause disease by gene truncation, but also by removing cis-acting regulatory elements, e.g. tissue-specific enhancers, from specific target genes (long-range position effects - LRPE). By comparing genomic features at the known LRPE-associated loci, we have defined hundreds of regions that could be at risk for LRPE. We confirm this by systematic whole genome mate-pair sequencing of ~300 two-way BCRs, including the first large cohort of healthy BCR carriers. Our concerted action will have the potential to establish a saturated map of genotype-phenotype links for hundreds of regulatory domains as high risk regions for LRPE, in addition to provide genotype-phenotype links for thousands of truncated protein-coding and non-coding genes.

NEXT GENERATION SEQUENCING (NGS) IN CARDIOLOGY

Špela Stangler Herodež[1], Danijela Krgović[1,2], Nadja Kokalj Vokač[1,2], Damijan Vokač[3]

[1]Medical Clinical Centre Maribor, Laboratory of Medical Genetics, Slovenia
[2]University of Maribor, Medical Faculty, Slovenia
[3]Medical Clinical Centre Maribor, Department of Cardiology, Slovenia

Introduction: The advent of next generation sequencing (NGS), in the last few years, has revolutionized the approach to genetics studies and has been shown to be successful in identifying novel causative mutations of rare or common Mendelian disorders. In cardiovascular medicine, NGS is used in the diagnosis of both Mendelian disorders of the cardiovascular system and complex genetic cardiovascular diseases (CVDs) including inherited cardiomyopathy, channelopathies, stroke and coronary artery disease. In all cases of a suspected genetic disorder, genetic testing is an important tool to help clarify and specify the precise diagnosis, and may be especially useful in cases of borderline clinical findings. **Material and Methods:** NGS analysis of genomic DNA was implemented on MiSeq platform. For that purpose the Illumina TruSight Cardio panel was used. We performed four runs including 48 patients with different cardiac phenotypes. Analysis and interpretation of the NGS data was done with Variant Studio (Illumina) software and free-access tools and databases. **Results:** NGS achieved 300x average of mean region coverage depth and yielded a coverage >10x in 99.3% targeted regions. In 7 patients pathogenic/probably pathogenic variants were found (Table). The variants of unknown significance (VOUS) were identified in 12 patients. On the other hand, the genetic cause of the pathology was not found in 29 patients. **Conclusions:** A rate of 15% positive genetic diagnoses highlights the effectiveness of NGS in the diagnosis of Mendelian disorders of the cardiovascular system and CVDs caused by a single variant in a single gene and in identifying novel causative mutations. A single test may identify the causative gene mutation in someone with an heart condition thereby allowing their relatives to be easily tested for the same gene mutations. The amount of data obtained during the NGS analysis also points out the need of precise clinical definition and better strategies for the determination of the pathogenicity of the identified variant and additional analyses are needed to further classify VOUS.

Table: Pathogenic/probably pathogenic variants.

Patient	Gene	Transcript change	Variant type
1	TTN	NM_001256850.1:c.69415C>T	stop-gain
2	LMNA	NM_170707.3:c.654delC	frameshift
3	DSP	NM_004415.2:c.273+1G>A	alternative splicing
4	PKP2	NM_004572.3:c.2453delG	frameshift
5	LMNA	NM_170707.3:c.654delC	frameshift
6	MYH6	NM_002471.3:c.2161C>T	missense
7	MYH7	NM_000257.2:c.1562T>C	missense

GENOMIC ANALYSIS IN CHILDHOOD SCHIZOPHRENIA

Danijela Krgović[1,2], Špela Stangler Herodež[1], Nina Šenica[3], Hojka Gregorič Kumperščak[3], Nika Nemec[4], Andreja Zagorac[1], Nadja Kokalj Vokač[1,2]

[1]University Medical Centre Maribor, Laboratory of Medical Genetics, Slovenia
[2]University of Maribor, Medical Faculty, Department for Molecular Biology, Slovenia
[3]University Medical Centre Maribor, Division of Paediatrics, Slovenia
[4]University of Ljubljana, Biotechnical faculty, Department of Animal Science, Chair of Genetics, Animal Biotechnology and Immunology, Slovenia

Introduction: Childhood schizophrenia is rare complex psychiatric disorder with polygenic inheritance. The therm Early Onset Schizophrenia (EOS) is used when symptoms are present after the age of 13 years. Before that age, children are diagnosed with Very Early Onset Schizophrenia (VEOS). Preliminary data suggest that childhood schizophrenia has higher genetic liability to the disease. Therefore, we preformed genetic testing using both molecular karyotyping (arrayCGH) and Next Generation Sequencing (NGS) for determination of Copy Number Variations (CNVs) and Single Nucleotide Variants (SNVs) affecting the possible schizophrenia-associated genes in a small group of Slovenian children diagnosed with EOS. **Material and Methods:** Our study included 36 patients. In all patients molecular karyotyping was performed using **SurePrint G3 Unrestricted CGH ISCA v2 8x60K** and 4x180K arrays, following the medical exome sequencing, which included 4813 genes (Illumina TruSight One capture), performed on the MiSeq sequencer. Data interpretation was carried out with Illumina Variant Studio and on-line available tools and databases. Considering the polygenicity of the disorder, we also analysed enrichment of the list of genes harbouring deleterious variants in Gene Ontology terms (GO) by using GeneMania version 3.4.1 Cytoscape plugin. **Results:** In 17% (6/36) of children a clinically significant CNV was detected, that could be associated with the disorder. In 25% (9/36) of patients SNV of unknown significance (VOUS) were detected, although no known pathogenic variants were identified for the selected genes. Therefore, using the GO terms, we assembled a list of genes harbouring probably deleterious variants. **Discussion:** The aim of this study was to assemble a list of genes harbouring probably pathogenic variants in Slovenian patients with EOS by performing the molecular karyotyping and NGS method. In order to achieve this goal we focused only on genes that are less tolerant to alteration and harbour variants that were predicted to be deleterious based on algorithms for deleteriousness detection and the type of mutation. Results of the performed enrichment analysis show that gene ontology terms related to schizophrenia are enriched in genes, selected by our filtering. The over-represented pathways could be associated with pathology of schizophrenia in Slovenian population.

EXPRESSION OF PRO-APOPTOTIC *miR-34a* AND ITS TARGET GENES IN HUMAN MYOCARDIAL INFARCTION AND HEALTHY HEART TISSUE

Emanuela Boštjančič[1], Maja Jerše[1], Nina Zidar[1]

[1]University of Ljubljana, Faculty of Medicine, Institute of Pathology, Department of Molecular Genetics, Slovenia

Apoptosis is an important mechanism in myocardial infarction (MI) as well as in heart development, being regulated or induced by numerous factors, including miRNAs. One of the most important pro-apoptotic miRNAs is p53-responsive *miR-34a*. In experimental model of MI, its function has been already shown as a circulating marker. However, its expression in human MI and in healthy heart tissue has not been elucidated yet. Based on our previous studies [1-3], we hypothesized that the expression of *miR-34a* and its target genes is similar in MI and in foetal hearts but different in healthy adult hearts.

Study included autopsy heart tissue samples of 45 patients with MI and heart tissue from healthy persons (10 adults, 5 children and 14 foetuses). We analysed expression of *miR-34a*, of its target genes (BCL2, ALDH2, PNUTS, SMAD4) and of caspase-3 by using real-time PCR. In a subset of MI patients, expression in infarcted tissue was compared to corresponding remote myocardium.

Expression of *miR-34a* was up-regulated in infarcted tissue and remote myocardium compared to healthy hearts. Expression of *miR-34a* in remote myocardium was up-regulated also when compared to corresponding infarcted heart tissue. Expression of *miR-34a* was higher in foetal hearts compared to healthy adults, although there was no significant difference between the two groups. The inverse correlation of *miR-34a* expression to the anti-apoptotic target genes was also observed. The highest expression of *miR-34a* was found in infarcted tissue 2-7 days after MI, and accordingly all the target genes showed the lowest expression 2-7 days after MI.

We compared these results to our previous study of immunohistochemically expressed caspase-3 [1-3] and M30 [2-3] as markers of apoptosis in human MI and foetal hearts, respectively. Based on our results, we suggest that elevated expression of *miR-34a* has, beside others, pro-apoptotic function in human MI as well as in the foetal hearts.

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HEREDITARY ANGIOEDEMA DUE TO C1-INHIBITOR DEFICIENCY: HETEROGENEITY OF *SERPING1* MUTATIONS, AND GENETIC FACTORS MODIFYING THE CLINICAL PHENOTYPE

Matija Rijavec[1], Peter Korošec[1], Slađana Andrejević[2], Ljerka Karadža-Lapić[3], Vesna Grivčeva-Panovska[4], Mitja Košnik[1,5]

[1]University Clinic of Respiratory and Allergic Diseases Golnik, Slovenia
[2]Clinical Center of Serbia, Clinic of Allergology and Immunology, Serbia
[3]General Hospital Šibenik, Croatia
[4]Dermatology Clinic, School of Medicine, Ss. Cyril and Methodius University, Republic of Macedonia
[5]Medical Faculty Ljubljana, Slovenia

Hereditary angioedema due to C1 inhibitor deficiency (C1-INH-HAE) is a rare autosomal dominant disease characterized by recurrent oedemas and large heterogeneity in clinical presentation, with an estimated prevalence of one case per 50,000 persons. The condition is caused by mutations in the *SERPING1* gene, which codes for C1-INH, located on chromosome 11q12-q13.1. More than 450 different mutations in *SERPING1* have been described to date, resulting in low levels of C1-INH (C1-INH-HAE type I) or normal levels of non-functional C1-INH (C1-INH-HAE type II). Our aim was to determine the spectrum of *SERPING1* mutations of C1-INH-HAE patients from Croatia, Republic of Macedonia, Serbia, and Slovenia. We also investigated if genetic factors, specifically type of *SERPING1* mutation and functional alterations *F12* and *KLKB1* affect the clinical phenotype of the disease. A cohort of 150 clinically well characterised C1-INH-HAE patients from 75 unrelated families from Croatia, Republic of Macedonia, Serbia, and Slovenia was recruited for genetic analysis, which included sequencing and MLPA analysis of *SERPING1*, as well as detection of *F12* (rs1801020) and *KLKB1* (rs3733402) polymorphisms. We have identified 39 different mutations; among them 14 missense, 11 nonsense, 7 frameshift, 1 in-frame deletion, 2 splicing defects, 1 substitution affecting the promoter, 2 large deletions and 1 large insertion. Thirteen mutations have not been previously described. In one patient only the homozygous variant c.-21T.C was found while in four patients no causative mutations could be identified. When addressing the phenotype-genotype relationship we found that patients with nonsense and frameshift mutations, large deletions/insertions, splicing defects, and mutations affecting the C1-INH active site (at Arg444) exhibited an increased clinical severity score based on the age of disease onset, organs affected and long-term prophylaxis, compared with those with missense mutations, excluding mutations at Arg444. In addition, the *F12* polymorphism was associated with disease onset. Our study identified 39 different, among them 13 novel, disease-causing mutations in C1-INH-HAE patients, highlighting the heterogeneity of mutations in the *SERPING1* gene. Furthermore, we have demonstrated on large number of patients that mutations with a clear effect on C1-INH function predispose patients to a more severe disease phenotype and the CC *F12* polymorphism predisposes patients to earlier disease onset.

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DETECTION OF SOMATIC MUTATIONS WITH ddPCR FROM LIQUID BIOPSY OF CRC PATIENTS

Uršula Prosenc Zmrzljak[1], Rok Košir[1], Zoran Krivokapić[2,3], Dragica Radojković[4] and Aleksandra Nikolić[4]

[1]Bia Separations CRO, Labena d.o.o, Molecular Biology Laboratory, Slovenia
[2]Clinical Center of Serbia, Department of Colorectal Diseases, Clinic for Digestive Surgery, First Surgical Clinic, Serbia
[3]University of Belgrade, Medical Faculty, Serbia
[4]University of Belgrade, Institute of Molecular Genetics and Genetic Engineering, Serbia

With the ever increasing use of biological drugs in cancer treatment, tumor mutation status has become an important part of molecular diagnostics that enables optimal treatment response. Genetic screens for somatic mutations, that arise spontaneously within tumor cells, are performed on genes including KRAS, BRAF and NRAS that are known to be frequently mutated in CRC tumors and confer resistance to therapies directed against EGF signaling pathway. Considering that these mutations provide a selective advantage for tumor cell survival, detecting somatic mutations present in wild type background with as low as < 0.1% is of potential diagnostic utility. Currently, the main source of tumor sample DNA for mutation detection is paraffin embeded tissue. However, in recent years liquid biopsy has become a promising alternative. Liquid biopsy is obtained from venous blood, which represents a source of cell free DNA (cfDNA) that can be isolated from either plasma or serum fractions. While the use of liquid biopsy is not included in current protocols for mCRC patient management, it has been extensively studied as a non-invasive method for both treatment response and patient monitoring. Its use in routine practice however needs to be technically and clinically validated (Montagut et al.).
The aim of the study was to determine the cfDNA levels and the KRAS and BRAF mutation status of a group of patients who underwent surgical removal of primary CRC. Since the source of cfDNA in the bloodstream can be of various origins (different necrotic / apoptotic processes), we compared cfDNA concentrations of CRC patients to two additional groups (hemorrhoid patients and healthy individuals). cfDNA was isolated from serum and its concentration measured with our custom designed human gDNA ddPCR assay which enables efficient and specific measurement of low concentrations of human DNA. The highest concentration of cfDNA was measured in CRC patients group (average 0.44 ng/ul), while both hemorrhoid patients and healthy individuals had significantly lower average concentrations, 0.25 ng/ul (* p = 0.01) and 0.08 ng/ul (p = 0.0001), respectively. Elevated cfDNA concentration can be an indicator of pathological or necrotic processes occurring in the body. However, these kinds of results must be interpreted with caution since other factors in addition to cancer can be the cause of elevated cfDNA serum concentrations. All patients were previously screened for somatic mutations in the 12 and 13 KRAS codons and the BRAF V600E mutation using the SNaPshot Multiplex System. Mutations were confirmed with Sanger sequencing (Nikolic et al. 2017). Mutations were detected in some of the primary tumors, but their presence in the serum was not confirmed with the use of the above mentioned method. In the current study, we detected the same mutations using BioRad's ddPCR mutation assays and showed that ddPCR is the method of choice when more sensitive measurements are required in molecular diagnostics in oncology.

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QUANTIFICATION OF HUMAN CYTOMEGALOVIRUS WITH DIGITAL PCR

Mojca Milavec[1]

[1]National institute of biology, Department of Biotechnology and Systems Biology, Slovenia

Quantitative PCR (qPCR) is an important tool in detection and quantification of human infectious agents. However, the use of different qPCR components, calibration materials and DNA extraction methods reduces comparability between laboratories, which can result in false diagnosis and discrepancies in patient care. In recent years, a novel version of PCR, known as digital PCR (dPCR), has become widely used in the field of nucleic acid analysis. As dPCR uses limiting dilutions and sample partitioning into sub-microlitre reactions, it can achieve more sensitive, precise, accurate, reliable and reproducible quantification of nucleic acids.
To investigate the potential role of dPCR in diagnostics of infectious agents, a method for quantification of human cytomegalovirus (HCMV) has been developed and compared to qPCR in terms of linearity, analytical sensitivity and limit of quantification. It has been shown that dPCR is a suitable method for robust and reproducible quantification of viral DNA that does not depend on a calibration material, has higher tolerance to inhibitors and only minor susceptibility to different PCR components that hamper qPCR efficiencies. Furthermore, dPCR potential, quantification of HCMV has been assessed in an inter-laboratory comparison on different dPCR platforms using whole virus material and extracted DNA. Although no calibrator has been used in the inter-laboratory comparison, discrepancies in estimated mean DNA copy number concentrations of viral DNA between laboratories and dPCR platforms were less than twofold, with DNA extraction as the main source of variability. This demonstrates that dPCR is beneficial in the field of clinical diagnostics, especially where inter-laboratory standardization of the qPCR-measured viral loads is limited by absence of standard materials. Moreover, dPCR has the potential for value assignment of materials for external quality assurance schemes and primary or secondary reference materials that are used for calibration of the widely used qPCR methods.

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Massimo Mezzavilla, Institute for Maternal and Child Health - IRCCS "Burlo Garofolo" – Trieste

Role of Natural Selection in Genetic Isolates: Possible advantages in complex-trait association studies

Cene Gostinčar, Department of Biology, Biotechnical Faculty, University of Ljubljana and Department of Molecular and Biomedical Sciences, Jožef Stefan Institute, Ljubljana, Slovenia

Genomics of Extremophilic Fungi: Answered and Unanswered Questions

Nataša Štajner, University of Ljubljana, Biotechnical Faculty, Ljubljana, Slovenia

The Potential of New NGS Approaches for Accurate Genotyping

Alenka Matjašič, Department of Molecular Genetics, Institute of Pathology, Faculty of Medicine, University of Ljubljana, Slovenia

lncRNAs as Regulators of Epigenetic Pathways in Glioma Development

Vita Šetrajčič Dragoš, Department of Molecular Diagnostics, Institute of Oncology, Ljubljana, Slovenia

The Importance of Functional mRNA Analysis in Clinical Variant Interpretation


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ROLE OF NATURAL SELECTION IN GENETIC ISOLATES: POSSIBLE ADVANTAGES IN COMPLEX-TRAIT ASSOCIATION STUDIES

Massimo Mezzavilla[1]

[1]Institute for Maternal and Child Health - IRCCS "Burlo Garofolo", Italy

Abstract

Population variation in disease liability and traits has been shaped by different factors, including environment, admixture events, bottlenecks and evolutionary history, which include natural selection including both positive and purifying selection. The adaptive variation that occurs because of natural selection usually act in a direct way on functional variants linked to phenotypic variation (1) and selected alleles usually confer protective effects (e.g. pathogen resistance) (2). Research in this area is also critical as the combination of genomic selection scan and association signals (GWAS) at a specific genetic locus in a specific population supports the presence of functional variation for a disease, as demonstrated in a study on coronary artery disease (3). In this light, genetic isolates have special features that can be exploited to increase the power of GWAS, as previously shown (4,5). Isolates have usually experienced bottlenecks and genetic drift, for this reason, some deleterious rare variants have increased in frequency while some neutral rare ones have decreased frequency. These two features are useful for the discovery of rare variant signals behind complex traits (6). Studies to date have focused on single isolates and have identified a good number of trait-associated signals (7,8). However, isolates might diverge in different aspects: time since the isolation, their initial founding size, the level of gene flow, and consequently they might differ in their power for GWAS (9). We can expect that only a small fraction of deleterious variants has increased in frequency in a precise isolate, so multiple isolates should be examined to uncover the total diversity of possible associated variants. Numerous pieces of evidence suggest relaxed purifying selection (removal of deleterious alleles) in the isolates due to their reduced population size (10) respect to the general reference population. This feature of less effectiveness of natural selection combined with genetic drift will be analyzed on whole genome data of different European isolated populations. Through the development of novel statistics such as DVxy and SVxy we demonstrate relaxation of purifying selection in the isolates (10), resulting in an enrichment of low-frequency deleterious variants. We also describe a novel indicator of isolation called isolation-index (Isx) that predicts several key genetic characteristics of an isolate and can help guide population choice for future complex-trait GWAS studies.

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GENOMICS OF EXTREMOPHILIC FUNGI: ANSWERED AND UNANSWERED QUESTIONS

Cene Gostinčar[1,2], Nina Gunde-Cimerman[1]

[1]University of Ljubljana, Biotechnical Faculty, Department of Biology, Slovenia
[2]Jožef Stefan Institute, Department of Molecular and Biomedical Sciences, Slovenia

With the increasing accessibility of next generation sequencing technologies, whole genome sequencing projects are no longer the exclusive domain of well-funded international consortia. As a consequence the sequencing of genomes of non-conventional model organisms has become a valuable tool in understanding the exceptional diversity of the living world. The sequencing of fungal genomes is of particular interest due to their larger complexity compared to prokaryotes but also smaller size than most other eukaryotes.

Our research of fungi from extreme environments identified species that can survive in conditions hostile to most other organisms, such as at high concentrations of salt, in some cases even in saturated solutions. Besides tackling basic questions, understanding the adaptation mechanisms of these species has substantial applicative potential. This past work enabled us to be the first to sequence the genomes of halophilic and extremely halotolerant fungi. The availability of the whole genome sequences facilitated further research on these species, which are often not an easy object for laboratory manipulation. It was clear from the start, however, that the analysis of a handful of genomic sequences alone would not be able to fully explain the unusual lifestyle of halophilic fungi. After sequencing the genomes of the extremely halotolerant black yeast *Hortaea werneckii*, the halophilic basidiomycete *Wallemia ichthyophaga* and four polyextremotolerant black yeast species *Aureobasidium* spp., we thus continued with sequencing additional strains of the same species – an effort providing us with a much more thorough insight into these peculiar organisms.

The lecture will summarize our genomic work on extremophilic fungi, with a focus on questions that can (and cannot) be answered with comparative and population genomics. Unusual problems that were encountered during our work and required tailor-made analytical approaches will be discussed and illustrated with examples from our published and ongoing research.

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THE POTENTIAL OF NEW NGS APPROACHES FOR ACCURATE GENOTYPING

Nataša Štajner[1], Urban Kunej[1], Valérie Laucou[2], Roberto Babilieri[2], Jernej Jakše[1]

[1]University of Ljubljana, Biotechnical Faculty, Slovenia
[2] UMR AGAP, Equipe Diversité et Adaptation de la Vigne et des Espèces Méditerranéennes, INRA, 2 Place Viala, France

Various international projects are focused on the preservation of genetic resources that are under risk of extinction. In some countries, mostly from the south-eastern Europe, level of information about the indigenous germplasm and their genetic diversity is still inadequate. This information is necessary for the management of germplasm collections as well as for conservation and assessment of variability. The ambiguities can be overcome by molecular characterization (e.g. using molecular markers), a very appropriate tool, which is, in contrast to phenotypic characterization, independent on environment conditions and has proven to be powerful tool for identity and kinship analysis in a variety of species.

The past projects on genotyping were mostly accomplished using multiplex PCR and capillary electrophoresis (CE) approaches based on identification of amplified length polymorphisms. The main challenge when using this methodology is the standardization of the allele sizes when comparing two data-sets. At this step, manual sizing and editing is required, which must be very precise to avoid false results. Besides, data analysed by CE technique, do not allow the determination of a full sequence of marker but limit the information to the length polymorphism, which also hinders straightforward comparison of the data sets. As an alternative, Next Generation Sequencing methods (NGS) offer information about DNA sequences including identification of their flanking regions. The advanced approach provides a deeper insight and more precise evaluation of allele variants applicable for sample identification and parentage analysis. We examine 1) the target enrichment via hybridization-based capture that provides an efficient and sensitive means for sequencing of specific genomic regions in a high-throughput manner and 2) the suitability of a semi-quantitative sequencing approach for microsatellite genotyping and the validation of the methodology by comparing the data generated by NGS to alternative obtained CE-based data. NGS sequencing approach facilitates high multiplexing of SSR loci and also enables identification of variations, which is limited when using conventional length polymorphism SSR genotyping. We believe that using NGS genotyping there is still scope for the improvement in the terms of speed, accuracy and price, especially when fusion of various data sets from different laboratories is needed to obtain large-scale study.

lncRNAs AS REGULATORS OF EPIGENETIC PATHWAYS IN GLIOMA DEVELOPMENT

Alenka Matjašič[1], Mara Popović[2] and Damjan Glavač[1]

[1]University of Ljubljana, Faculty of Medicine, Institute of Pathology, Department of Molecular Genetics, Slovenia
[2]University of Ljubljana, Faculty of Medicine, Institute of Pathology, Slovenia

ABSTRACT

Molecular-genetic analyses are becoming an important part in cancer diagnostics. Identifying genetic drivers can help diagnose glioma tumours in their early stages. Gliomas are clinically extremely heterogeneous tumours, presenting a great challenge in clinical oncology. There is numerous evidence that disturbance of epigenetic mechanisms contributes to cell's malignant transformation and cancer progression. One of key epigenetic modulators, known to be involved in tumorigenesis of different tumours types, including glioma, are long non-coding RNAs (lncRNA). These RNAs are regulators of crucial protein-coding genes (mRNA) on multiple levels and of various signalling pathways. Aberrant gene expression is already shown to be in correlation with specific tumour type, and several comparative expressional studies showed we can arrange glioma in different subtypes upon similarities in their expression patterns. Our main purpose was to determine gliomas´ expression profiles and search for novel potential (non)coding biomarkers of glioma development. Using the microarray technology, we screened a smaller cohort of glioma biopsy samples for defining expressional differences of 879 lncRNAs and their 477 potential mRNA targets, which are involved in epigenetic signalling pathways. Many coding and non-coding genes showed significantly aberrant expression (390 and 351, respectively), and the number varied among the glioma subtypes as tumours of higher malignancy grade showed higher expressional heterogeneity. In search of new potential biomarkers, we performed an integrative analysis of lncRNA-mRNA expression and identified 400 matched pairs for 184 differentially expressed lncRNAs and 171 predicted mRNAs (Figure 1). Gene-annotation enrichment analysis and functional annotation of mRNA targets using DAVID tools showed their association, and thus of lncRNAs, mainly with transcription processes and chromatin modifications/remodelling.

In conclusion, involvement of lncRNAs in disease processes creates an urgency to understand how much the disturbance of their expression and functions might contribute to disease development. lncRNAs are probably just as important as mRNA in glioma development and progression, and taking all into consideration, gliomas are not only clinically, but also (epi)genetically extremely heterogeneous entities. Expressional analyses provide us framework for further analyses of (epi)genetic mechanisms underlying glioma formation and progression.

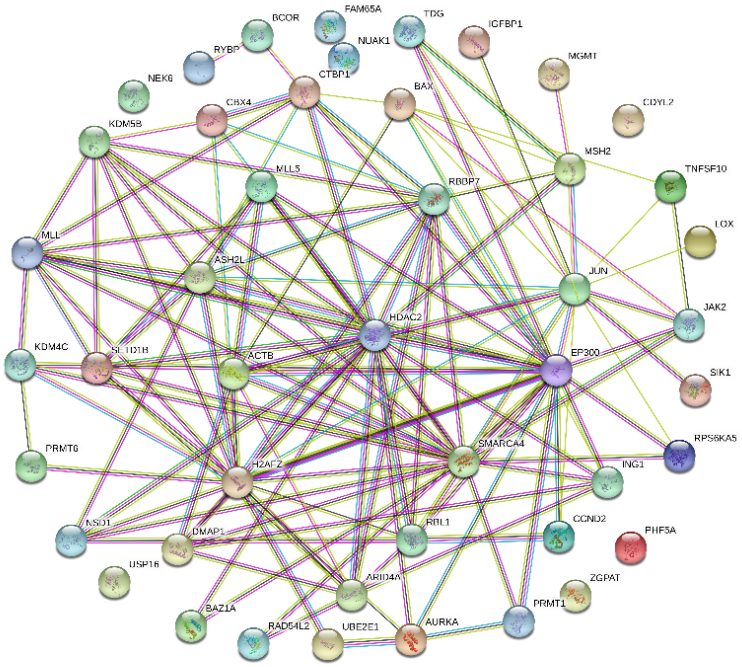

Figure 1. Protein-protein interaction network (STRING-db) of potential lncRNA-associated mRNA targets in primary glioblastoma, obtained by integrative analysis of lncRNA-mRNA expression patterns.

THE IMPORTANCE OF FUNCTIONAL mRNA ANALYSIS IN CLINICAL VARIANT INTERPRETATION

Vita Šetrajčič Dragoš[1], Gašper Klančar[1], Vida Stegel[1], Ana Blatnik[2], Mateja Krajc[2], Srdjan Novaković[1]

[1] Institute of Oncology, Department of Molecular Diagnostics, Slovenia
[2] Cancer Genetics Clinic, Institute of Oncology, Slovenia

With the breakthrough of NGS in molecular diagnostics, one of the arising problems is the identification of variants of uncertain clinical significance (VUS). VUS are problematic since are not clinically informative and are causing confusion among professionals and patients. Therefore it is necessary for clinical laboratories to implement functional assays for further classification of VUS. Here we present a case of a female 54 year old, which was referred to genetics screening for germline NF1 mutation. DNA was extracted from whole venous blood and all exons with flanking 25bp of intronic regions of NF1 gene was sequenced using NGS. No known pathogenic variant was identified, however the analysis revealed a new missense variant in exon 2 (*NF1*:c.122A>T p.?), distanced 60bp from 3' splice site and 83bp from 5' splice site. Splicing *in silico* tools predicted the variant to cause a strong *de novo* donor splice site. To further asses the variant's impact on pre-mRNA splicing, whole mRNA was extracted from blood and direct Sanger sequencing was performed from 5'UTR to exon 5. Transcript analysis revealed the disruption of canonical donor splice site by *de novo* donor splice site, causing a 84bp deletion of exon 2. Due to functional mRNA analysis, the variant of uncertain clinical significance *NF1*:c.122A>T was reclassified to a likely pathogenic variant. In conclusion, our result strongly supports the strategy to functionally asses all identified VUS for which *in silico* tools predict alteration of pre-mRNA splicing.


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Bojana Žegura, Department of Genetic Toxicology and Cancer Biology, National Institute of Biology, Ljubljana, Slovenia

3D Cell Cultures and their Application in Genetic Toxicology

Jernej Jakše, Department of Agronomy, Biotechnical Faculty, University of Ljubljana, Slovenia

Ten Years of Citrus Bark Cracking Viroid in Hop Gardens

Metka Ravnik Glavač, Institute of Biochemistry, Faculty of Medicine, University of Ljubljana and Department of Molecular Genetics, Institute of Pathology, Faculty of Medicine, University of Ljubljana, Slovenia

Epigenetics of mind-body interactions

Urška Sivka, Omega d.o.o., Ljubljana, Slovenia

Identification by Transcriptome Analysis of Genes Associated with Chemical Boring in the Date Mussel (*Lithophaga lithophaga*)

Aleš Sedlar, Agricultural Institute of Slovenia, Crop Science Department, Slovenia

Transcription Profiling of PVY^{NTN} Susceptible Potato Tubers in Necrosis Suppressing and Promoting Storage Conditions


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3D CELL CULTURES AND THEIR APPLICATION IN GENETIC TOXICOLOGY

Bojana Žegura[1], Martina Štampar[1] and Metka Filipič[1]

[1]National Institute of Biology, Department of Genetic Toxicology and Cancer Biology, Slovenia

Hepatic cell-based assays are very important in the drug development process as well as safety assessment of chemicals to provide simple, fast, and cost-effective tools to avoid large-scale and cost-intensive animal testing. Most of the cell-based assays use traditional two-dimensional (2D) monolayer cells that however have several limitations and have poor correlation with *in vivo* conditions. The major limitations are the lack of numerous biological functions like cell–cell and cell–matrix contacts, which results in decreased cell differentiation, flattened morphology of cells with altered cytoskeleton, reduced viability, and altered cell signalling pathways and most importantly reduction or loss of many hepatic enzymes involved in metabolism of xenobiotic substances. Therefore, it is very important and essential to develop improved *in vitro* cell-based systems that can more realistically mimic the *in vivo* cell behaviours and provide more predictive results to *in vivo* conditions. In this respect three-dimensional (3D) cell culture systems have gained increasing interest in drug discovery and tissue engineering due to their evident advantages in providing more physiologically relevant information and more predictive data for *in vivo* tests compared to 2D culture system. The 3D models have improved cell-cell and cell-matrix interactions and have preserved complex *in vivo* cell phenotypes. Moreover, 3D hepatic models exhibit higher level of liver-specific functions including metabolic enzymes compared to 2D models. Therefore, tremendous effort has been put into the development of a variety of 3D models, which hold the promise for applications in drug discovery, cancer cell biology, stem cell research, safety studies and many other cell-based analyses by bridging the traditional 2D cell culture models monolayer cell culture and whole-animal systems. The presentation will give an overview of hepatic 3D cell cultures and their potential application in genetic toxicology.

TEN YEARS OF CITRUS BARK CRACKING VIROID IN HOP GARDENS

Jernej Jakše[1], Nataša Štajner[1], Ajay Kumar Mishra[2], Jaroslav Matoušek[2], Tanja Guček[3], and Sebastjan Radišek[3]

[1]University of Ljubljana, Biotechnical Faculty, Department of Agronomy, Slovenia
[2] Institute of Plant Molecular Biology, Biology Centre ASCR v.v.i, Czech Republic
[3] Slovenian Institute of Hop Research and Brewing, Slovenia

In 2017, hop grower in Savinja valley complained about the appearance of severely stunted hop plants. This phenomenon spread rapidly within hop fields and among farms, indicating mechanical mode of transmission within field or same farm and diseased plant material between farm. High-throughput parallel sequencing (NGS) of total RNA and small RNAs from plants with and without symptoms was employed and reads were assembled by *de-novo* algorithm or mapped to reference viral/viroid genomes. Novel sequence of Citrus bark cracking viroid (CBCVd) was identified as casual agent and confirmed in control transmission experiment. This new viroid in hops presented a challenge for farmers and authorities to prevent its spread and to eradicate infested plants, as well to develop a fast and reliable method for its detection. A reliable and robust one-step multiplex RT-PCR (mRT-PCR) was developed to simultaneously detect several hop viroids. To understand the nature of To investigate possible PTGS mechanisms of viroids *in-silico*, prediction of target hop transcripts for HLVd and CBCVd derived small RNAs were performed. Prediction models revealed that 1062 and 1387 hop transcripts share nucleotide homologies with HLVd- and CBCVd-derived small RNAs, respectively. Therefore, they could be silenced in the RNAi process. Expression profiles of selected transcripts mainly showed expression fluctuations compared to viroid-free plants, with possible evidence of down-regulation of two transcripts. To study the response of the hop micro RNA genes during CBCVd infection, identification of 116 miRNAs from the hop genome was performed. Many of them were found to be differentially expressed in response to CBCVd infection. Targets for these miRNAs are also transcriptional factors that may regulate important hop processes. A powerful tool for studying gene expression on a global scale is available through high-throughput sequencing of RNA or RNA-seq. Recently, we have initiated a RNA-seq experiment to address the issue of hop gene expression alterations by single and mixed viroid infections. We believe that efforts to achieve better understanding of the molecular mechanisms of the viroid diseases are needed to initiate novel strategies against these diseases and help to discover genetic resistance.

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EPIGENETICS OF MIND-BODY INTERACTION

Metka Ravnik-Glavač[1,2]

[1] University of Ljubljana, Faculty of Medicine, Institute of Biochemistry, Slovenia
[2]University of Ljubljana, Faculty of Medicine, Institute of Pathology, Department of Molecular Genetics, Slovenia

ABSTRACT

There are lengthy and still unresolved debates in philosophy, religion, psychology, and cognitive science about what is mind. Older viewpoints included dualism and idealism considered the mind somehow non-physical, while modern mainly Western views often center around physicalism and functionalism, which investigate the mind in relation to the physical brain and neuronal activity.
The brain is the central organ for adaptation to experiences, including stressors, which are capable of changing brain architecture as well as altering systemic functions. Chronic stress or inefficient management of stress can activate both the ANS and the HPA axis and lead to inflamation, which can, in turn, cause cardiovascular, neurological, metabolic and psychiatric diseases.
Meditation practices have spread in Western countries as a safe and efficacious remedy to counteract distress. Numerous research studies suggest that yogic/meditative practices have significant positive effects on the mind–body system and can thereby increase wellness in health and support prevention and healing from diseases.
Epigenetic mechanisms represent the bridge between environmental inputs and ever-changing patterns of gene expression. Stress has been associated with changes in epigenetic mechanisms as well as in expression of genes. Recent studies show evidence of rapid gene expression changes in chromatin regulatory enzymes and DNA methylation enzymes, as well as alterations in histone modifications and DNA methylation patterns after meditation practices and thus provide the molecular biology evidence of mind-body interaction.

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IDENTIFICATION BY TRANSCRIPTOME ANALYSIS OF GENES ASSOCIATED WITH CHEMICAL BORING IN THE DATE MUSSEL (*LITHOPHAGA LITHOPHAGA*)

Urška Sivka[1], Nataša Toplak[1], Simon Koren[1], Jernej Jakše[2]

[1]Omega d.o.o., Slovenia
[2]University of Ljubljana, Biotechnical Faculty, Slovenia

Lithophaga lithophaga is a rock-boring bivalve with an ability to dissolve carbonate substrata with its siphonal and mantle tissue secretions. Three different methods for dissolution of the substrate have been proposed: (i) secretion of mucoproteins with calcium-binding ability (Jaccarini et al. 1968), (ii) secretion of glycolipoproteins with phosphate groups (Bolognani et al. 1976), and (iii) excretion of metabolic carbon dioxide (Lazar and Loya 1991). The genetic background that enables *L. lithophaga* to bore into calcareous rocks is not yet known. In order to identify genes potentially involved in chemical boring by *L. lithophaga* we compared mantle tissue transcriptomes in *L. lithophaga* with those in *Mytilus galloprovincialis*, which lacks the ability to bore into rocks. Transcriptome sequencing of *L. lithophaga* and *M. galloprovincialis* using an Ion Torrent platform generated 60,563 and 75,216 million clean reads with an average read length of 96 bp and 108 bp, respectively. *De novo* assembly of clean reads of *L. lithophaga* produced 62,490 contigs with a mean length of 408 bp. Sequence annotation against six databases resulted in 42,587 transcripts of *L. lithophaga* with a gene description, protein domain or gene ontology term. Since the boring mechanism is attributed to calcium-binding proteins, the search focused on transcripts capable of binding calcium. In all, 178 genes with calcium-binding ability were found to be expressed in the pallial gland of *L. lithophaga*. Five of these genes (*annexin B9-like isoform x5*, *sarcoplasmic calcium-binding protein*, *calreticulin*, *phospholipase* A_2 and *V-type* H^+*-ATPase*) were considered as possible candidate chemical-boring genes and their relative expression was investigated in four different tissues with qPCR method. The results from gene expression analysis revealed that *calreticulin*, *phospholipase* A_2 and *sarcoplasmic calcium-binding protein* were up-regulated in the pallial gland. Two of the candidate genes (*annexin B9-like isoform x5* and *V-type* H^+*-ATPase*) had the same expression profile in all four investigated tissues. The results of this study shed light on the genetic background of the chemical boring mechanism in date mussel. This set of candidate genes could be used in future research studies of chemical boring in the date mussel and other marine or freshwater chemical borers.

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TRANSCRIPTION PROFILING OF PVY^{NTN} SUSCEPTIBLE POTATO TUBERS IN NECROSIS SUPPRESSING AND PROMOTING STORAGE CONDITIONS

Aleš Sedlar[1], Barbara Gerič Stare[2], Irena Mavrič Pleško[2], Peter Dolničar[1], Marko Maras[1], Jelka Šuštar-Vozlič[1], Špela Baebler[3], Kristina Gruden[3], Vladimir Meglič[1]

[1]Agricultural Institute of Slovenia, Crop Science Department, Slovenia
[2]Agricultural Institute of Slovenia, Plant Protection Department, Slovenia
[3]National Institute of Biology, Department of Biotechnology and Systems Biology, Slovenia

The necrotic strain of Potato virus Y (PVY^{NTN}) is one of the most damaging potato pathogens and can also affect the quality of harvested tubers of secondary infected plants. Cold postharvest storage of potato tubers at 4°C effectively reduces and delays PVY^{NTN}-associated necrosis formation[1]. The aim of our study was to investigate the response of PVY^{NTN}-infected potato tubers on a biological pathway level during necrosis suppressing (4°C) and promoting (transfer to 25°C) conditions.
Healthy and infected potato tubers of susceptible cultivar Igor stored under different conditions were assessed for virus concentration using qPCR and global transcriptional changes using potato genome microarrays. Metabolic pathway analysis was performed utilizing MapMan plant ontology adapted for potato[2] and software tools MapMan and GSEA.
During cold storage necrosis formation in infected tubers was completely suppressed, no increase in virus concentration was observed and the effect of infection decreased with storage duration. The effect of cold storage duration on the onset time and percentage of necrosis after tuber transfer to 25°C was also reflected on the host transcription level. Only a small number of infection-associated differentially expressed genes were observed in tubers that did not develop necrosis while the highest changes were observed in the necrotic tuber tissue.[3] Among the later several differentially expressed genes associated with programmed cell death were detected, such as those coding for proteins with caspase-like activity, endoplasmic reticulum chaperones and folding proteins, implicating the presence of vacuolar cell death and unfolded protein response. Potato tuber transcription profiles were additionally evaluated in a meta-analysis of several studies of potato/pathogen interactions and resemblance between the systemic necrosis in susceptible tubers and the hypersensitive response of primary infected plants was observed implying on the resemblance of susceptible plant response to the hypersensitive response of resistant plants.
The study will contribute to the understanding of potato/PVY interactions leading to necrosis formation, as well as the study of specific processes, such as plant programmed cell death.
The study was supported by the Slovenian Research Agency (projects L4-2400-0401, J4-7636, programmes P4-0072, P4-0165), the FP7 Project CropSustaIn (FP7-REGPOT-CT2012-316205) and the World Federation of Scientists (National Scholarship).

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Zlatko Šatović, University of Zagreb Faculty of Agriculture, Department of Seed Science and Technology and Centre of Excellence for Biodiversity and Molecular Plant Breeding, Croatia

Genetic Diversity and Evolutionary History of *Salvia officinalis* L. and *Salvia lavandulifolia* Vahl

Jana Murovec, University of Ljubljana, Biotechnical Faculty, Ljubljana, Slovenia

Genome Editing of Plants and its Implementation for Breeding *Brassica* Species

Jelka Šuštar Vozlič, Agricultural Institute of Slovenia, Crop Science Department, Slovenia

Safety, Regulatory and Social Challenges Associated with Modern Biotechnology and Synthetic Biology

Gašper Žun, Jožef Stefan Institute, Department of Molecular and Biomedical Sciences and Faculty of Chemistry and Chemical Technology, Ljubljana, Slovenia

A Novel CRISPR-Cas9 Approach in Yeast *Saccharomyces cerevisiae* for Precise Allele Swap Allows Quantitative Trait Genes Validation

Michael Hansen, Qiagen, Denmark

Genomic Services Solutions at Qiagen: Detection of Exosome-Derived microRNAs

Špela Kos, Institute of Oncology Ljubljana, Department of Experimental Oncology, Slovenia

Synergy of DNA Vaccine with CTLA-4/PD-1 Blockade

Urša Lampreht Tratar, Institute of Oncology Ljubljana, Department of Experimental Oncology, Slovenia

The Increase of Immune Cell Infiltration after Interleukin 12 Gene Electrotransfer in Murine and Canine Tumors


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GENETIC DIVERSITY AND EVOLUTIONARY HISTORY OF *Salvia officinalis* L. AND *Salvia lavandulifolia* Vahl

Zlatko Šatović[1,2], Danijel Škrtić[3], Ivan Radosavljević[2,3], Marija Jug-Dujaković[4], Filip Varga[1,2], Llorenç Sáez[5], Romeo di Pietro[6], Zlatko Liber[2,3]

[1]University of Zagreb Faculty of Agriculture, Department of Seed Science and Technology, Svetošimunska cesta 25, Croatia
[2]Centre of Excellence for Biodiversity and Molecular Plant Breeding, Croatia
[3]University of Zagreb, Faculty of Science, Division of Biology, Department of Botany and Botanical Garden, Croatia
[4]Institute for Adriatic Crops and Karst Reclamation Split, Croatia
[5]Universitat Autònoma de Barcelona, Facultat de Biociències, Spain
[6]Sapienza Università di Roma, Dipartimento di Pianificazione, Design e Tecnologia dell'Architettura, Italy

Dalmatian sage (*Salvia officinalis* L.) and Spanish sage (*S. lavandulifolia* Vahl) are closely related, perennial species from the family *Lamiaceae*. Dalmatian sage is native to coastal regions of the western Balkans as well as to the southern Apennine Peninsula while the natural distribution area of Spanish sage is the Iberian Peninsula. Thus, the populations of Dalmatian and Spanish sage most probably survived in different refugia during the glacial periods according to the traditional interpretation of southern European peninsulas (Balkans, Apennines and Iberia) as being single refugia that served as a source for a postglacial recolonization. However, 'refugia within refugia' model implies the existence of multiple glacial refugia within each larger refugial area that may confound the interpretation of phylogeographic patterns. Microsatellite markers and chloroplast DNA sequences were used to examine and compare genetic diversity and population structure of Dalmatian and Spanish sage with the aim to assess the fine-scale geographical distribution of putative 'refugia within refugia'.

Key words: *Salvia officinalis*, *Salvia lavandulifolia,* microsatellites, chloroplast DNA, 'refugia within refugia', Balkan peninsula, Apennine peninsula, Iberian peninsula.

GENOME EDITING OF PLANTS AND ITS IMPLEMENTATION FOR BREEDING *BRASSICA* SPECIES

Jana Murovec[1], Katja Guček[1], Borut Bohanec[1], Monika Avbelj[2], Roman Jerala[2]

[1]University of Ljubljana, Biotechnical Faculty, Slovenia
[2]National Institute of Chemistry, Department of synthetic biology and immunology, Slovenia

CRISPR/Cas9-mediated genome editing is revolutionizing life sciences by providing new, easy-to-use, precise and high-throughput tools for genetic modification through induction of target specific DNA doubled stranded breaks. It is used for basic and applied genetics of the majority of agronomically important plant species, including species of the genus *Brassica*. However, the *A. tumefaciens*-mediated delivery method used so far (Lawrenson *et al.*, 2015; Braatz *et al.*, 2017; Kirchner *et al.*, 2017; Yang *et al.*, 2017) gives rise to concerns about its regulatory status, since the use of transgenesis during variety development can trigger GMO regulations in countries that rely on process-based regulatory approaches.
The aim of our work was therefore to develop a DNA-free genome editing system for cultivars of the species *B. rapa* and *B. oleracea*. We developed an efficient protocol for isolation of viable protoplasts and for the introduction of ribonucleoproteins (RNPs) composed of preassembled Cas9 enzyme and *in vitro* transcribed sgRNA. Two endogenous genes were targeted: 1. the phytoene desaturase gene (*PDS*) involved in the carotenoid biosynthesis pathway and 2. the vernalization determinant *FRIGIDA* (*FRI*) gene. Several sgRNA were designed for each of the two genes, *in vitro* transcribed and their cleavage efficiency tested with an *in vitro* digestion assay. Two sgRNAs per gene were selected and used for further experiments on the PEG-mediated delivery of RNPs into protoplasts. One to three days after transfection, total plant DNA was isolated from protoplasts, the regions surrounding the targeted sequences were amplified by PCR and analyzed by amplicon deep sequencing and T7EI assay. The results showed that mutation induction frequencies were species, gene, and locus dependent and correlated with the introduced concentrations of RNPs. They showed the feasibility of RNPs mediated targeted mutagenesis of *B. rapa* and *B. oleracea* for DNA- and selectable marker-free genome editing.
The results will be discussed in regard to their applicability for plant breeding, other genome editing studies that are currently in progress and our future research activities.

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SAFETY, REGULATORY AND SOCIAL CHALLENGES ASSOCIATED WITH MODERN BIOTECHNOLOGY AND SYNTHETIC BIOLOGY

Mojca Milavec[2], Marko Dolinar[3], Luka Juvančič[4], Aleš Sedlar[1], Jelka Šuštar-Vozlič[1]

[1]Agricultural Institute of Slovenia, Crop Science Department, Slovenia
[2]National Institute of Biology, Slovenia
[3]University of Ljubljana, Faculty of Chemistry and Chemical Technology, Slovenia
[4]University of Ljubljana, Biotechnical Faculty, Slovenia

The techniques of modern biotechnology, introduced in the 1970s with the first successful cloning of bacterial genes and transfer of foreign DNA between different organisms, shorten the process of modifying the genetic material in different fields of applications, ranging from plant breeding to pharmaceutical production. A comprehensive biosafety system has been established to monitor their application and release of products on the market and into the environment. It is aimed at effective control over the use of recombinant DNA technology and its products, genetically modified organisms (GMOs), in order to ensure a high level of protection of human and animal health and the environment from the potentially harmful effects of these technologies.
In the last decade, the existing framework of biosafety has been faced with new challenges. Recent scientific advances in the field of modern biotechnology have enabled the development of simpler, more reliable and suitable techniques for modifying the genetic material. Another relatively new scientific discipline - synthetic biology has been developed at the intersection of biology and engineering. Methodologically it is based on recombinant DNA technology; however it surpasses simple combining of two or more genetic elements and enables genetic rearrangement and subsequent modification of protein circuits in the cell.
These new techniques raise several issues from the biosafety and regulatory point of view to different social issues, international trade, and possibility of consumer choice, the protection of natural resources or food security. Due to all these, the existing systems and approaches need to be re-evaluated in the context of biosafety. Particular attention should be given to environmental and human health risk assessment, the need for monitoring as well as anticipated potential socio-economic impacts.
New techniques of modern biotechnology and synthetic biology as well as the existing legislation in the field of GMOs have been reviewed, with special emphasis given to environmental risk assessment. The applicability of existing approaches for risk assessment of products of modern biotechnology and synthetic biology is being assessed with a view to ensure a high level of biosafety. In the course of the project, appropriate amendments of risk assessment of new techniques will be proposed if needed. In addition, a set of socio-economic factors associated with wide use of new techniques has been defined. Together, these results will contribute to the discussion of eventual improvements of the biosafety regulatory system in light of new techniques of biotechnology and synthetic biology.

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A NOVEL CRISPR-Cas9 APPROACH IN YEAST *Saccharomyces cerevisiae* FOR PRECISE ALLELE SWAP ALLOWS QUANTITATIVE TRAIT GENES VALIDATION

Gašper Žun[1,2], Mojca Ogrizović[1], Uroš Petrovič[1,3]

[1]Jožef Stefan Institute, Department of Molecular and Biomedical Sciences, Slovenia
[2]Faculty of Chemistry and Chemical Technology, University of Ljubljana, Slovenia
[3]Biotechnical Faculty, Department of Biology, University of Ljubljana, Slovenia

The majority of complex traits are polygenic and to find corresponding quantitative trait loci (QTL) or even quantitative trait genes (QTG), statistically and genetically relevant population has to be inspected. However, to narrow the quantitative trait area, to confirm and to validate it, requires a more mechanistic concept.
To assess a single gene as a QTG within a predicted QTL for the variability in a complex trait, allele swap has to be performed and phenotype re-evaluated. Double-strand brakes that are created by an endonuclease system CRISPR-Cas9 in the budding yeast are preferentially repaired via homologous recombination, thus offering this repair mechanism a designated matrix with adequate homology regions will result in precise genome editing. This encouraged us to swap between suspected alleles of QTG in otherwise isogenic background.
Despite high level of performance of the CRISPR-Cas9 technique we came across two limitations in allele swapping. First, because of high level of sequence similarity between the two alleles, strand invasion can occur anywhere within the allele, and not necessarily between distance points used as homologous regions. Secondly, after the exogenous DNA, which is used as a matrix for homologous recombination, had degraded, the double strand break is repaired via non-homologous end joining. We solved the latter limitation by introducing point mutations in the linear PCR-amplified matrix, which corresponds to the protospacer adjacent motif (PAM) sequence. Consequently, Cas9 could not recognize its DNA target after the allele swap had been achieved. The former limitation was solved using two distinctive sequential CRISPR-Cas9 systems, whereby the new allele is inserted into the region of the previously deleted homologous allele. An example will be shown where QTG for neutral lipid storage in *S. cerevisiae* was swapped in this way.
This is the first demonstration that a fast, efficient and precise two-step approach using CRISPR-Cas9 could be performed to successfully swap alleles in otherwise isogenic backgrounds, possibly also for massive genome manipulation. Such an approach will be of great value for further dissection of QTGs, as it provides precise perturbations in a selected gene of the same segregant.

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GENOMIC SERVICES SOLUTIONS AT QIAGEN: Detection of Exosome-derived microRNAs

Michael Hansen[1]

[1]Qiagen, Denmark

Exosomes function as shuttles for transferring functional molecules such as proteins, liquids and nucleic acids between donor and recipient cells. As natural carriers of signal molecules i.e. microRNAs, these tiny nano-sized (30-150 nm) vesicles play crucial roles in the progression of several human diseases, including neurodegenerative disease, cancer, cardiovascular disease and inflammatory diseases. There is no doubt we are at the era of discovering novel cell-free therapeutic and diagnostic biomarkers to deliver on the promise of individualized, patient-specific therapies for the future. Given the small size and heterogeneity of exosomes, it is very challenging to isolate exosome-derived microRNAs from blood plasma and other body fluids.
QIAGEN Genomic Services are now making it even easier for customers to get started with their exosome-derived microRNA projects having optimized the complete workflow from initial sample isolation to extensive data analysis, assistance with data interpretation and design of downstream validation studies. Here we show the results of the exosome-derived microRNA NGS product which has been optimized to work with QIAGEN chemistry and processes. We compared ExoRNeasy kit and the miRNeasy S/P kit to isolate RNA combined with our very sensitive QIAseq library prep kit for microRNAs. The protocol developed by Genomic Services gives highly reproducible data achieving usable sequencing libraries from as little as 200 µl input material. However, the less input material we utilize the higher library prep failure we observe, the more primer-dimers are present in the sequencing data and the more technical variable the results become. Thus, we recommend to use minimum 500 µl serum or plasma as starting material. With 500 µl and 1 ml input, we see robust call rates of 400-500 miRNAs expressed at above the threshold of 1 TPM and 150-200 miRNAs expressed above 10 TPM.

SYNERGY OF DNA VACCINE WITH CTLA-4/PD-1 BLOCKADE

Špela Kos[1], Alessandra Lopes[2], Veronique Preat[2], Maja Čemažar[1,3], Gregor Serša[1,4], Gaelle Vandermeulen[2]

[1]Institute of Oncology Ljubljana, Department of Experimental Oncology, Slovenia
[2]Université Catholique de Louvain, Louvain Drug Research Institute, Advanced Drug Delivery and Biomaterials, Belgium
[3]University of Primorska, Faculty of Health Sciences, Slovenia
[4]University of Ljubljana, Faculty of Health Sciences, Slovenia

DNA vaccines and immune checkpoint inhibitors hold a great promise in prevention and treatment of a variety of cancer types. However, neither of the two approaches have fully met expectations in cancer therapy when used as a single treatment. Therefore, we aimed at exploring whether the combination of intradermal DNA vaccination and immune checkpoint blockade exert synergistic antitumor effectiveness in murine melanoma tumor model.
We observed that dual blockade of CTLA-4 and PD-1 immune checkpoint receptors enhanced B16F10-OVA tumor rejection in mice immunized against ovalbumin or melanoma antigen gp100. Compared to single treatments, a combination of intradermal DNA vaccination and immune checkpoint blockade resulted in a significant antigen-specific immune response, with higher production of antigen-specific IgG antibodies and increased intratumoral CD8+ infiltration. Strong activation of the immune response induced by combined treatment resulted in significant delay of tumor growth and prolonged survival of treated mice.
We demonstrated that dual CTLA-4/PD-1 blockade and DNA vaccination work hand in hand to improve tumor immunity and contribute to significant anti-tumor response in melanoma-bearing mice. These results indicate on the potential application of the combined DNA vaccination and immune checkpoint blockade, specifically, to enhance the efficacy of DNA vaccines and to overcome the resistance to immune checkpoint inhibitors in certain cancer types.

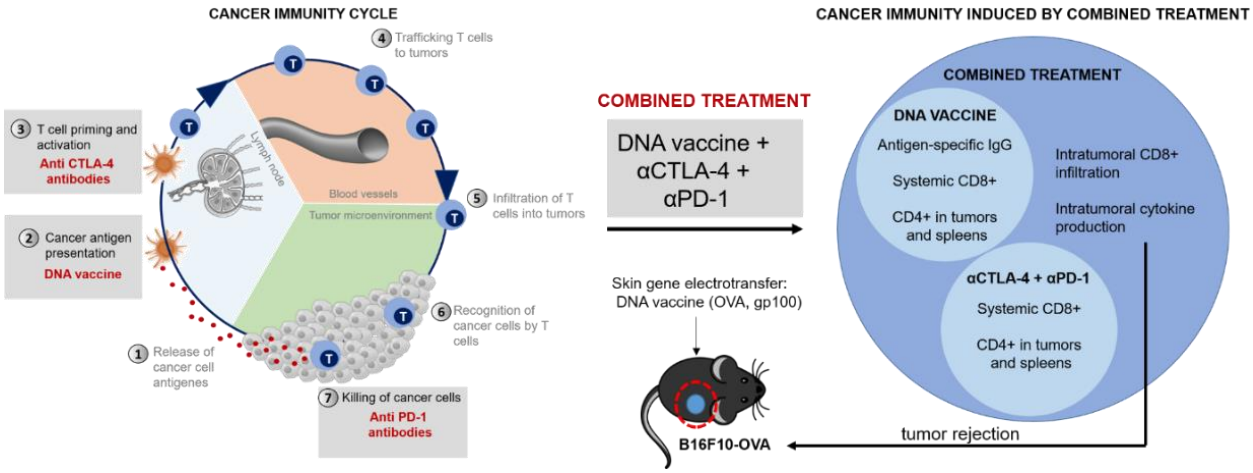

Figure: Cancer immunity induced by combined treatment of DNA vaccines and immune checkpoint inhibitors.

THE INCREASE OF IMMUNE CELL INFILTRATION AFTER INTERLEUKIN 12 GENE ELECTROTRANSFER IN MURINE AND CANINE TUMORS

Urša Lampreht Tratar[1], Urška Kamenšek[1], Luisa Loiacono[2], Emanuela Signori[2,3], Gregor Serša[1], Maja Čemažar[1,4]

[1]Institute of Oncology Ljubljana, Department of Experimental Oncology, Slovenia
[2]Laboratory of Genetic and Clinical Pathology, University Campus Bio-Medico of Rome, Italy
[3]Laboratory of Oncology, IRCCS "Casa Sollievo della Sofferenza", Italy
[4]University of Primorska, Faculty of Health Sciences, Slovenia

Interleukin-12 (IL-12) is a potent stimulator of proliferation and chemotaxis of immune cells. Gene electrotransfer studies using plasmids encoding IL-12 have shown great promise in treating tumors induced in experimental animals[1] and also in human and veterinary clinical studies.[2,3] The aim of this study was to evaluate the immune response after IL-12 gene electrotransfer in murine and canine tumors. Gene electrotransfer of IL-12 was performed in C57Bl/6 mice bearing B16F10 tumors by murine IL-12 plasmid and in nude mice bearing CMeC-1 canine tumors by canine IL-12 plasmid. Both plasmids encoded IL-12 under constitutive promotor and had no antibiotic resistance gene. Our results showed that IL-12 gene electrotransfer resulted in complete eradication of B16F10 tumors and in a statistically significant prolonged tumor growth delay of CMeC-1 tumors compared to control group. The local immune response was evaluated by immunohistochemical staining of F4/80 and MHCII (M1 macrophages) and CD11c (dendritic cells) in B16F10 tumors and granzyme B (cytotoxic cells) staining in CMeC-1 tumors. The results demonstrated higher infiltration of M1 macrophages and dendritic cells in the B16F10 tumors and higher infiltration of cytotoxic cells in CMeC-1 tumors. Besides local response, systemic response was also evaluated by measurement of IL-12 and IFNγ in blood serum with ELISA assay. In both cases, an evident systemic response was present as demonstrated by a statistically significant increase in IL-12 and IFNγ in blood serum. This study demonstrates promising results in targeting tumor microenvironment by IL-12 gene electrotransfer.

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D. Phillip Sponenberg, Virginia Maryland College of Veterinary Medicine, Virginia Tech, Blacksburg, VA, USA

Genetic Aspects of Conserving Local Breeds

Simon Horvat, University of Ljubljana, Biotechnical faculty, Department of Animal Science, Slovenia

The Endangered Slovenian Drežnica Goat Breed: Assessing Genotypic-Phenotypic Diversity and Population Structure

Peter Dovč, University of Ljubljana, Biotechnical Faculty, Department of Animal Science, Slovenia

Global Biodiversity in Domestic Pig Breeds (*Sus scrofa*)

Mojca Ogrizović, Jožef Stefan Institute, Department of Molecular and Biomedical Sciences, Slovenia

Elucidating the Pleiotropic Effects of *MKT1* Gene in Yeast *Saccharomyces cerevisiae*

Barbara Pipan, Agricultural Institute of Slovenia, Crop Science Department, Slovenia

Marker-Assisted Evaluation and Trait-Specific Selection of Accessions from Central and Eastern European Common Bean Germplasm


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GENETIC ASPECTS OF CONSERVING LOCAL BREEDS

Sponenberg D. Phillip[1]

[1]Virginia Maryland College of Veterinary Medicine, Virginia Tech, USA

Conservation of local breeds of livestock is an international priority identified by the FAO. The genetic aspects of local breed populations affect their rational use and conservation. Two important aspects of the genetics of these populations is their status and role within their species, and also the structure and organization of the individual population targeted for conservation. The general genetic organization of domesticated animals loosely follows the original development and expansion that each of them took out of their original center of domestication. Each expansion was a foundation event that sampled a portion of the original variation, such that peripheral and local populations tend to represent different portions of the original genome than do the international breeds commonly used for production. Certain geographic areas contain more variation than do others, and also contain different variants than others. For some species (cattle, horses) the wild ancestors are now extinct, and therefore conservation of the domesticated descendants is all that can be done to assure that these taxa do not become extinct. For other species with extant wild ancestors, the genetic variation of the domesticates remains distinct from the wild ancestors and therefore also warrants conservation. Successful conservation of these breed populations is a process of discovering them, securing them, and sustaining them. This needs to be accomplished by attention to the combination of factors that led to breeds: foundation, isolation, and selection imposed both by the natural environment and the human owners of the resource. Genetic structure of populations leads to different strategies for effective conservation. Production breeds usually have a pyramidal structure with elite animals dominating the genetics of the population. Sampling the elites therefore captures most of the genetic variation of the population. In contrast, local breeds usually have a structure of genetically isolated subpopulations. Sampling each of these is necessary to fully capture the genetic variation of the population. Targeted sampling, rather than a random approach, can result in more complete representation of the population's genome. In most local breeds the majority of variation is present in females, especially older females that may predate any bottleneck in the history of the breed's census. Breeding protocols must account for this in order to not lose this variation. Common protocols that use single males extensively for long breeding careers result in great loss of variation. In contrast, using younger males for only single seasons results in increased variation. Selecting replacement males from older females is especially effective in retaining the variation present in that portion of the population.

THE ENDANGERED SLOVENIAN DREŽNICA GOAT BREED: ASSESSING GENOTYPIC-PHENOTYPIC DIVERSITY AND POPULATION STRUCTURE

Simon Horvat[1], Ivica Medugorac[2], Dominik Pečovnik[1], Tim Goričan[1], Danijela Bojkovski[1], Mojca Simčič[1]

[1]University of Ljubljana, Biotechnical faculty, Department of Animal Science, Slovenia
[2]Faculty of Veterinary Medicine, Germany

Genetic research in domestic animals has important scientific and socio-economic effects in the field of medicine, health and welfare of domestic animals and agriculture in general. Domestic animals are especially interesting for scientific research because they are the source of extreme phenotypic variability. For all autochthonous ruminant breeds in Slovenia, and especially for Drežnica goat breed, phenotypes are extremely adapted to poor and rough environment and very extensive rearing technology. Due to the artificial selection of breeders to certain traits and, on the other hand, of parallel-acting natural selection, especially on survival traits, Drežnica goat presents a unique source of genetic and phenotypic variability. This breed suffered strong population decrease after the second world war and is today at high risk of extinction due to small population size (629) and because the majority of population is concentrated within a radius of less than 30 km in Slovenian western Alps. The main objective of this project was to evaluate the genetic authenticity of the breed as well as genetic relationship to other cosmopolitan and geographically closer breeds. Genome-wide Single Nucleotide Polymorphism (SNP) and haplotype analyses were performed on 96 unrelated Drežnica goats and 577 animals from 13 reference populations representing ten Alpine goat breeds from Switzerland and three Angora breeds as outgroups. Drežnica goat branched out as a distinct breed in phylogenetic analyses with a very high number of private alleles. Genetic distance and ThreeMix matrix analyses confirmed an independent origin of the breed. Multivariate outlier test detected only a small number of outliers. Parentage testing using microsatellite markers determined that 44.4% (8 out of 18) of pedigrees contained incorrect parent data and helped to assign some unknown sires to the offspring. Ongoing are also studies in characterizing coat color pigments and patterns. Preliminary analyses reveal a very high diversity of coat color and patterns in Drežnica goat. Another ongoing research examines a hypothesis about the possibility and extent of the wild ancestral genome of Alpine ibex (*Capra ibex*) introgression into the Drežnica goat genome. In conclusion, Drežnica goat represents an excellent model for studies of mechanisms related to the search for genetic bases for the determination of phenotype and the evolution of genetic variants and adaptive properties. Such analyses are also essential in preservation efforts of this breed in its original type.

GLOBAL BIODIVERSITY IN DOMESTIC PIG BREEDS (*Sus scrofa*)

Peter Dovč[1], Minja Zorc[1], Jernej Ogorevc[1], Luca Fontanesi[2], Alan Archibald[3], Meta Čandek Potokar[4]

[1]University of Ljubljana, Biotechnical Faculty, Department of Animal Science, Slovenia
[2]University of Padua, Department of Animal Genetics, Italy
[3]Roslin Institute, Edinburg, United Kingdom
[4]Agricultural institute of Slovenia, Department of Animal production, Slovenia

Pigs were domesticated from wild boars independently in Asia and Europe about 10,000 years ago. Asian and European pigs differ significantly due to the differences among ancestral populations, which have been geographically separated for about 1.2 million years. From Europe and Asia, *Sus scrofa* was introduced to other continents, North Africa, North- and South America, Australia and New Zealand. Due to selection for different traits, a multitude of pig breeds around the world, varying in coat colour, ear shape, body size, growth rate, fatness, prolificacy and other economically important traits, has been established. Modern genotyping methods allow a profound insight into population structure, detection of migrations, formation of historical bottlenecks and detection of selection signatures in these populations. In this study, we used porcine 60K SNP chip for high throughput genotyping of more than 120 pig breeds and several wild boar populations worldwide. Our data reflect key events related to domestication, dispersal and crossing of pigs from different genetic backgrounds. If the imports of Chinese pigs to Europe, mainly to United Kingdom, were characteristics of the late 18th century, than represent the recent imports of European pigs to China during the last decades, closing of the globalisation circle. The detection of Runs of Homozygosity (ROH) revealed the effects of inbreeding, due to limited size of local populations and selection pressure for different economically important traits in numerous populations. Our special focus was on several local breeds in Mediterranean area and on the Balkan Peninsula, especially in relation to geographic isolation and inbreeding. These, until recently untapped breeds, represent an important genetic reservoir for development of new breeding strategies towards better meat quality and more sustainable pork production. Results of our study reveal important facts about this segment of agrobiodiversity and provide necessary data for conception of genetic conservation measures in order to preserve the existing genetic richness of different pig breeds.

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ELUCIDATING THE PLEIOTROPIC EFFECTS OF *MKT1* GENE IN YEAST *SACCHAROMYCES CEREVISIAE*

Mojca Ogrizović[1], Peter Kogovšek[1,2], Andreja Petrič[1], Uroš Petrovič[1,2]

[1]Jožef Stefan Institute, Department of Molecular and Biomedical Sciences, Ljubljana, Slovenia
[2]University of Ljubljana, Biotechnical Faculty, Department of Biology, Slovenia

Mkt1 is yeast protein that functions as a posttranscriptional regulator of many stress-related transcripts. It has been found as a quantitative trait gene in quantitative trait loci (QTL) mapping studies conducted against the S288c reference laboratory strain. The *MKT1*^{S288c} allele is rare in the *S. cerevisiae* population. It contains two variants, G30D and R453K, which are found almost exclusively in the S288c strain. It has been proposed that the *MKT1*^{S288c} is a loss-of-function allele and that the G30D mutation is the causal mutation. The allele has been found to be disadvantageous under several stressful conditions, with the exception of the addition to the medium of rapamycin or haloperidol, where *MKT1*^{S288c} has been found to be beneficial. Nevertheless, most of the studies have been limited to only one non-S288c strain, and the effect of *MKT1*^{S288c} in different genetic backgrounds is still not proven. We conducted a growth profiling screening experiment in different *S. cerevisiae* strains. Using end point colony size method and plate dilution assay we measured the growth of strains at several different stressful environmental conditions. In line with previous studies, in all tested conditions and backgrounds the *MKT1*^{S288c} behaved as a loss-of-function allele, with the 30D variant being causal. For the resistance to rapamycin, however, we showed that all the non-S288c alleles, having the 30G variant, were inferior, regardless of the strain. We next determined the phenotype of 15 segregants from the S288c × AWRI1631 cross. We found that additional alleles are involved in the resistance to rapamycin and that their presence determines the positive or negative effect of the *MKT1*^{S288c} allele for this trait. In the case of caffeine resistance, we showed that *MKT1* is involved in the molecular mechanism behind this trait, and that the S288c allele is superior. Our study is the most comprehensive systematic study where the effect of *MKT1* allele variant and its point mutations were experimentally studied in different *S. cerevisiae* strains. It also importantly contributes to the understanding of the pleotropic character of the *MKT1* gene.

MARKER-ASSISTED EVALUATION AND TRAIT-SPECIFIC SELECTION OF ACCESSIONS FROM CENTRAL AND EASTERN EUROPEAN COMMON BEAN GERMPLASM

Barbara Pipan[1], Lovro Sinkovič[1], Vladimir Meglič[1]

[1]Agricultural Institute of Slovenia, Crop Science Department, Slovenia

Common bean (*Phaseolus vulgaris* L.) is the most important edible food legume for direct human consumption in the world as it represents a valuable source of components with nutritional and health benefits. Thus, it is a crop featured in breeding programs worldwide, including Agricultural Institute of Slovenia. Genetic diversity of different common bean genetic resources has been evaluated in numerous studies using different DNA markers. Information on genetic diversity of common bean from Central and Eastern Europe is scarce; therefore our study was a basis for construction common bean core collection (CC) and final collection (FC) using Central and East European germplasm. Regarding to basic multi-crop passport descriptors and seed characteristics including geographic origin, biological status, ancestral data, phenotypic seed characteristics and different phaseolin type (corresponding Andean/Mesoamerican origin), 782 accessions (ACCs) from 9 gene banks and 12 geographic origins were selected and observed as a whole collection (WC). Genotyping procedure using 33 genome-specific nSSR and EST-SSR markers was performed to assess genetic background of WC. Regarding to diversity parameters and genetic structure, we proposed CC encompassing 63 accessions, representing global genetic diversity of WC - all of 782 ACCs from different geographic origins. Additionally, we identified 14 standard genotypes/known varieties expressing desirable traits of interest to construct final CC. Altogether, 77 genotypes from CC were further evaluated on different levels combining agronomic, genetic and selected nutritional traits of importance including: I) morphological/phenotypical evaluations to test actual agronomic traits/response including abiotic/biotic resistance potential under field conditions; II) morphometric analysis of seeds regarding to Community Plant Variety Office-Technical Protocol*/Phaselieu* common bean seed descriptors; III) marker-assisted evaluation and trait-specific selection for economically important traits (bean common mosaic virus, bean rust, anthracnose and angular leaf spot resistance; drought/heat tolerance; low phosphorus uptake and root morphology; high zinc and iron content; earliness and high yield) applying 75 trait-related DNA markers including KASP_SNP markers (24), nSSRs (35), SCAR (5), EST-SSR (2), CAPS (1) and locus-specific PCR based markers (8); IV) biochemical analysis including multi-elemental composition, fats, proteins and phytic acids. After marker-assisted evaluation and complex combination of the results obtained from all four evaluation levels using rating system, we established FC of 12 elite common bean ACCs representing superior potential for breeding applications.


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Sabina Semiz, International University of Sarajevo, Faculty of Engineering and Natural Sciences, Genetics and Bioengineering Program and University of Sarajevo, Faculty of Pharmacy, Bosnia and Herzegovina

Pharmacogenomics and Precision Medicine in Type 2 Diabetes

Uroš Potočnik, University of Maribor, Faculty of Medicine, Center for Human Molecular Genetics and Pharmacogenomics, Slovenia

Molecular mechanisms of response to biological therapy anti TNF by integrating system medicine and functional genomics approachees

Katja Goričar, University of Ljubljana, Faculty of Medicine, Institute of Biochemistry, Pharmacogenetics Laboratory, Slovenia

Association of *MSLN* Polymorphism with Serum Mesothelin Levels and Survival in Malignant Mesothelioma

Katja Repnik, Faculty of Medicine, University of Maribor, Centre for Human Molecular Genetics and Pharmacogenomics and Faculty of Chemistry and Chemical Engineering, University of Maribor, Laboratory of Biochemistry, Molecular biology and Genomics, Slovenia

Circulating Tumor DNA in Head and Neck Cancer as Predictive and Prognostic Molecular Marker

Nataša Debeljak, University of Ljubljana, Faculty of Medicine, Institute of Biochemistry, Slovenia

Exploring the Genetic Background of Familial Erythrocytosis


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PHARMACOGENOMICS AND PRECISION MEDICINE IN TYPE 2 DIABETES

Sabina Semiz[1,2], Tanja Dujic[2], Zelija Velija Asimi[3], and Adlija Causevic[2]

[1]International University of Sarajevo, Faculty of Engineering and Natural Sciences, Genetics and Bioengineering Program, Bosnia and Herzegovina
[2]University of Sarajevo, Faculty of Pharmacy, Bosnia and Herzegovina
[3]University of Sarajevo, Faculty of Medicine, Bosnia and Herzegovina

An enhanced understanding of genes and pathways that determine response to oral antidiabetic drugs (OAD) has the potential to reveal new drug targets, develop novel drugs, and optimize treatment of Type 2 diabetes (T2D) with promotion of more personalized approach. The most recent and promising advances in precision medicine of T2D are related to therapy with metformin, a first-line drug used to treat newly diagnosed diabetic patients. Recent studies reported an association of genetic variations in drug - metabolizing enzymes (DME), drug transporters (DT), and specific drug targets with T2D treatment outcomes in diverse population groups. Here we present results of our recent study in which we analyzed several common variants of OCT1 (*SLC22A1),* OCT2 *(SLC22A2),* MATE1 (*SLC47A1), STK11,* and *ATM* genes in 92 newly diagnosed T2D patients who were prescribed metformin as their initial hypoglycaemic therapy. Our results suggested associations of *STK11, ATM,* and *SLC47A1* polymorphisms with variability in the therapeutic response to metformin. Close to 50% of T2D patients experienced gastrointestinal side effects during the first six months of metformin treatment. We showed the association of *OCT1* reduced-function variants with the incidence of common metformin-induced gastrointestinal side effects in T2D patients. In conclusion, our results suggested an association between specific genetic polymorphisms and interindividual variability in metformin response. These results could contribute to more precise and safer treatment with metformin where genotype is used to guide diabetes therapy.

MOLECULAR MECHANISMS OF RESPONSE TO BIOLOGICAL THERAPY ANTI TNF BY INTEGRATING SYSTEMS MEDICINE AND FUNCTIONAL GENOMICS APPROACHES

Uroš Potočnik [1,2] Katja Repnik [1,2], Mario Gorenjak[1], Gregor Jezernik[1], Boris Gole[1] and Silvo Koder[3]

[1]University of Maribor, Faculty of Medicine, Center for Human Molecular Genetics and Pharmacogenomics, Slovenia
[2]University of Maribor, Faculty for Chemistry and Chemical Engineering, Laboratory for Biochemistry, Molecular Biology and Genomics, Slovenia
[3]University Medical Centre Maribor, Slovenia

Proinflammatory cytokine tumor necrosis factor-α (TNF-α) plays a dominant role in pathogenesis of chronic immune diseases, including rheumatoid arthritis (RA) and inflammatory bowel disease (IBD) (which includes Crohn's disease (CD) and ulcerative colitis (UC)). Biological drugs capable of blocking it, including infliximab (IFX) and adalimumab (ADA) are currently the leading treatment of several autoimmune conditions including RA and CD and could induce remission in up to 70% not responding to standard treatment or developing adverse drug effects, mainly to immunomodulators and corticosteroids. While development of antibodies against biological anti-TNF drugs could at least partially explain long term non-response, the mechanism of particularly short term non-response is poorly understood. The aim of our proposed study is to describe molecular mechanisms contributing to non-response to anti-TNF therapy in patients with CD and RA and to identify molecular genetic markers that could predict anti-TNF response, which could be used for personalized/precision medicine. We have developed biobank of biological samples (DNA, RNA, proteins, serum, colon biopsies) from CD and RA patients on ant-TNF therapy collected before and during treatment (weeks 0, 4, 12, 20, 30). Biobank is equipped with comprehensive biochemical data (24 parameters) and clinical data (34 items) including treatment response measured with IBDQ and CRP. We are conducting systems medicine approach by integration of genetic (NGS, genotyping), gene expression (RNAseq), proteomic, epigenetic and gene regulation (noncoding RNA profiling, splice variants) data with bioinformatic tools (gene ontology - GO) for identification and description of biological pathways important in anti-TNF treatment. We have developed functional cell model based on co-culture of immune and intestinal cells to conduct TNF inhibition *in vitro* to further confirm and more deeply describe the molecular pathways identified on our biobank samples representing TNF inhibition *in vivo*. Predictive models are being developed using machine learning algorithms. We have already proved the autophagy plays role in ADA response in CD patients, with *ATG16L1* and *ATG5* as the most promising biomarkers in this pathway. Other most promising pathways involved in anti TNF response include NF-κB, IL23, TLR and TNF/TNFR2 signaling, apoptosis, platelet activation and proteasome protein degradation. Currently, best described molecular mechanism of non-response to anti TNF therapy in CD is through interaction between IL23 and TNF/TNFR2 signaling. In responders, apoptosis of CD4+ T cells is induced by inhibition of membrane TNF on CD14 macrophages and preventing interaction between CD14+ macrophage membrane TNF and CD4+ T cells TNFR2 receptor. In non-responders, CD14+ macrophages produce IL23 which binds to CD4+ T cells IL23R receptor and prevent apoptosis of CD4+ T cells through STAT3 activation.

ASSOCIATION OF *MSLN* POLYMORPHISM WITH SERUM MESOTHELIN LEVELS AND SURVIVAL IN MALIGNANT MESOTHELIOMA

Katja Goričar[1], Alenka Franko[2], Metoda Dodič-Fikfak[2], Viljem Kovač[3], Vita Dolžan[1]

[1]University of Ljubljana, Faculty of Medicine, Institute of Biochemistry, Pharmacogenetics Laboratory, Slovenia
[2]University Medical Center Ljubljana, Clinical Institute of Occupational Medicine, Slovenia
[3]Institute of Oncology Ljubljana, Slovenia

Introduction: Exposure to asbestos is associated with increased risk for several diseases, including malignant mesothelioma (MM), a very aggressive cancer with poor survival. Biomarkers enabling early detection of MM and biomarkers of treatment response could improve prognosis of MM patients. Cell surface glycoprotein mesothelin is overexpressed in several tumors and studies have proposed serum mesothelin levels could be used for screening for MM in asbestos-exposed individuals. However, considerable interindividual variability in serum levels limits mesothelin's usefulness. As genetic factors could contribute to the observed variability, our objective was to assess the influence of *MSLN* rs1057147 (c.*69G>A) polymorphism within the putative miRNA-binding site on serum mesothelin levels and on survival of MM patients.
Materials and methods: In total, 154 MM patients were included in our study and serum samples at diagnosis were available for 86 patients. Patients were genotyped for *MSLN* rs1057147 using competitive allele-specific PCR, while commercial ELISA assay was used to determine serum mesothelin levels. Nonparametric Mann-Whitney test and Cox regression were used in statistical analysis.
Results: The median (range) serum mesothelin levels at MM diagnosis were 2.43 (0.0 to 34.8) nmol/l. Carriers of at least one polymorphic *MSLN* rs1057147 allele did not have significantly higher median serum mesothelin levels (2.62 vs 1.79 nmol/l, P=0.595). Median progression-free survival of MM patients was 7.8 (5.5-13.6) months, while median overall survival was 18.0 (9.8-29.6) months. Carriers of at least one polymorphic *MSLN* rs1057147 allele had significantly shorter overall survival compared to carriers of two wild type alleles (15.1 vs 22.2 months, HR=1.72, 95% CI=1.15-2.55, P=0.008). They also tended to have shorter progression-free survival (7.1 vs 9.3 months, HR=1.39, 95% CI=0.98-1.98, P=0.068).
Conclusions: *MSLN* genetic variability may be independently associated with survival of MM patients even though it cannot explain all the variability in serum mesothelin levels at diagnosis.

CIRCULATING TUMOR DNA IN HEAD AND NECK CANCER AS PREDICTIVE AND PROGNOSTIC MOLECULAR MARKER

Katja Repnik[1,2], Ellen Heitzer[3], Bogdan Čizmarević[4], Uroš Potočnik[1,2]

[1] University of Maribor, Faculty of Medicine, Centre for Human Molecular Genetics and Pharmacogenomics, Slovenia
[2] University of Maribor, Faculty for Chemistry and Chemical Engineering, Laboratory for Biochemistry, Molecular Biology and Genomics, Slovenia
[3] Medical University of Graz, Institute of Human Genetics, Diagnostic and Research Center for Molecular BioMedicine, Austria.
[4] University Medical Centre Maribor, Department of Otorhinolaryngology, Cervical and Maxillofacial Surgery Slovenia

Head and neck cancer represents increasing incidence and mortality in both developing and developed countries. In head and neck cancer the biomarkers that enable diagnosis of disease or monitoring of pre- and posttreatment tumor burden are not available. Thus, head and neck cancer surveillance relies on clinical and radiological findings. Liquid biopsies, e.g. the analysis of circulating tumor DNA (ctDNA), represent an alternative to tissue biopsies to characterize tumor genetic features in a non-invasive manner and is therefore very promising prognostic and predictive biomarker in a variety of cancer types. However, there are limited data relating head and neck cancer and ctDNA

In a proof-of principle study, we analyzed plasma DNA from 29 head and neck cancer patients using the mFAST-SeqS method, a fast and cost-effective determination of tumor DNA levels. In 24.1 % of patients, we identified ctDNA fractions of 10% or higher (*z*-score ≥5). Interestingly, in most of these patients their tumors were located in hypopharynx or larynx and of stage T3 or higher. In samples with *z*-scores greater than 5 we additionally performed genome-wide copy number profiling using plasma-Seq. We found a recurrent focal amplification on chromosome 11q13.3 which includes genes *CCND1* and *FADD*, which was also present in corresponding tumor tissue and represents one of the most prominent genetic alterations in head and neck cancer associated with poor prognosis.

We showed that ctDNA analysis can be applied in head and neck cancer and may be used as a valuable predictive and prognostic molecular biomarker. As genome-wide copy number analysis requires tumor fractions of 5% and higher, as with many other entities, a prescreening of tumor fraction might be useful to inform about tumor DNA level.

EXPLORING THE GENETIC BACKGROUND OF FAMILIAL ERYTHROCYTOSIS

Tadej Pajič[1], Martina Fink[1], Saša Anžej Doma[1], Irena Preložnik Zupan[1], Tanja Kunej[2], Nataša Debeljak[3]

[1]University Medical Center Ljubljana, Clinical department of Haematology, Slovenia
[2]University of Ljubljana, Biotechnical Faculty, Department of Animal Science, Slovenia
[3]University of Ljubljana, Faculty of Medicine, Institute of Biochemistry, Slovenia

Objectives: Familial erythrocytosis (FE) is a rare inherited blood disorder with heterogeneous genetic background. Currently there are nine genes associated with FE (*EPOR, EGLN1, EPAS1, VHL, EPO, HBA1, HBA2, HBB, BPGM*), but there are only five types of FE (ECYT1-5) classified within Online Mendelian Inheritance in Man (OMIM) compendium. Over 60% cases of FE are still of unknown etiology, which implies the existence of more genes are yet to be discovered.
The aims of our study are to determine frequencies of erythrocytosis in Slovenian population and genetic variations with indication for FE. We gather known information on sequence variants of genes that are associated with FE in aim to expand the current classification of erythrocytosis.
Results: Review of the clinical data from April 2011 till May 2018 indicated seven families with symptoms of FE. Literature review indicated several sequence variants in genes associated with FE (*EPOR, EGLN1, EPAS1, VHL, EPO* and *HBB*). Most of those are located on or close to regions that are responsible for protein-protein interactions. Analysis of listed genes with Sanger sequencing revealed few variants currently not associated with FE that needs to be functionally characterized. We are currently developing analysis with next generation sequencing (NGS).
Conclusions: Review of sequence analysis on patients with FE in Slovenia is currently undergoing.

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Ana Dolinar, University of Ljubljana, Faculty of Medicine, Department of Molecular Genetics, Institute of Pathology, Slovenia

Circular RNAs in Amyotrophic Lateral Sclerosis

Anja Pavlin**,** University of Ljubljana, Biotechnical Faculty, Department of Biology, Slovenia

Lytic Gene Expression in the Temperate Bacteriophage GIL01 is activated by a Phage-Encoded LexA Homologue

Ester Stajič, University of Ljubljana, Biotechnical Faculty, Department of Agronomy, Slovenia

CRISPR/Cas9-Mediated Genome Editing in Cabbage

Gregor Jezernik, University of Maribor, Faculty of Medicine, Slovenia

Fatty Acid Abnormalities in IBD are linked to Novel IBD Specific Loci

Helena Volk, Biotechnical Faculty, Department of Agronomy, Slovenia

The Invisibility Cloak: Chitin Binding Protein of *Verticillium nonalfalfae* Disguises Fungus from Plant Chitinases

Ida Djurdjevič, University of Ljubljana, Biotechnical Faculty, Department of Animal Science, Slovenia

Transcriptome Analysis and Cellular Background of the Pigment Pattern in Marble and Brown Trout

Katja Molan, University of Ljubljana, Biotechnical Faculty, Department of Biology, Slovenia

The *Escherichia coli* Colibactin Resistence Protein ClbS is a Novel DNA Binding Protein that Protects DNA from Nucleolytic Degradation

Katja Uršič, Institute of Oncology Ljubljana, Department of Experimental Oncology, Slovenia

Peritumoral Gene Electrotransfer of Interleukin-12 as Immune Boost to Intratumoral Electrochemotherapy for Treating Murine Melanoma

Kristina Marton, Biotechnical Faculty, Department of Agronomy, Slovenia

Potential Avirulence Gene Vna8.691 of Phytopathogenic Fungus Verticillium nonalfalfae

Luka Predojević, University of Ljubljana, Biotechnical Faculty, Department of Biology, Slovenia

Genetic, Biofilm and Cellular Analysis of Uropathogenic Virulence Factors of Various Human *Escherichia coli* Strains Employing a Biomimetic Urothelial Model *in vitro*

Sara Redenšek, University of Ljubljana, Faculty of Medicine, Institute of Biochemistry, Pharmacogenetics Laboratory, Slovenia

Genetic Variability in Oxidative Stress Pathways Influences the Occurrence of Adverse Events of Dopaminergic Treatment in Parkinson's Disease


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CIRCULAR RNAs IN AMYOTROPHIC LATERAL SCLEROSIS

Ana Dolinar[1], Damjan Glavač[1], Metka Ravnik-Glavač[1,2]

[1]University of Ljubljana, Faculty of Medicine, Department of Molecular Genetics, Institute of Pathology, Slovenia
[2]University of Ljubljana, Faculty of Medicine, Institute of Biochemistry, Slovenia

ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease with affected upper and lower motor neurons. The disease is partially explainable by several different genetic mutations among which are most frequent hexanucleotide expansions in *C9orf72* gene [1]. However, our understanding of disease initiation and its causes is still limited. Various epigenetic changes were already implied in the initiation and development of ALS [2]. Recently, circular RNAs (circRNAs) have been recognized as an important group of regulatory non-coding RNAs. We analysed the expression of 13 617 human circRNAs (Arraystar Human circRNA Arrays V2 (8x15K)) in RNA samples of 12 ALS patients and 8 healthy controls (performed by Arraystar Inc, USA). Hierarchical clustering of analysed circRNAs is shown in Figure 1. We identified 274 up-regulated and 151 down-regulated circRNAs in samples versus controls (fold-change in expression: > 1,5 and p-value < 0,05). When comparing male samples versus male controls we found 983 up-regulated and 1096 down-regulated circRNAs. The number of differentially expressed circRNAs in female samples versus female controls was much lower (5 up-regulated and 14 down-regulated circRNAs). Among these significantly up- or down-regulated circRNAs we selected 10 circRNAs for further validation on a cohort of 100 ALS samples. Selection criteria were fold-change in expression, potential involvement of circRNA in the pathogenesis of ALS and gene function if circRNA is intragenic.

To our knowledge, this is the first study on the expression of circular RNAs in human blood samples of patients with amyotrophic lateral sclerosis.

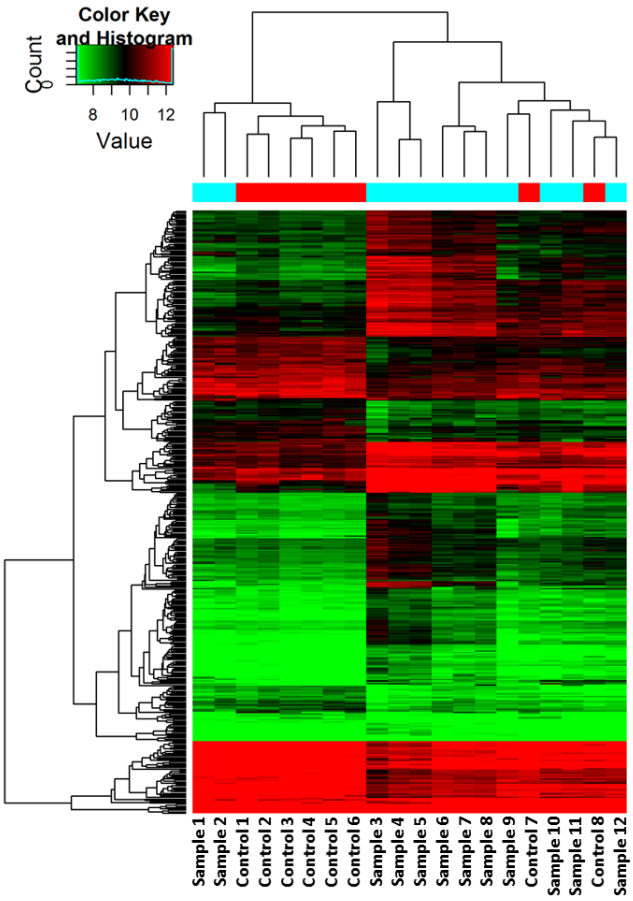

Figure 1: Hierarchical clustering of human circRNAs in samples of amyotrophic lateral sclerosis and healthy controls.

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LYTIC GENE EXPRESSION IN THE TEMPERATE BACTERIOPHAGE GIL01 IS ACTIVATED BY A PHAGE-ENCODED LexA HOMOLOGUE

Anja Pavlin[1], Nadine Fornelos[2,4], Douglas F. Browning[3], Zdravko Podlesek[1], Vesna Hodnik[1], Margarita Salas[2], Matej Butala[1]

[1]University of Ljubljana, Biotechnical Faculty, Department of Biology, Slovenia
[2]Instituto de Biología Molecular 'Eladio Viñuela' (CSIC), Centro de Biología Molecular 'Severo Ochoa' (CSIC-Universidad Autónoma de Madrid), Spain
[3]University of Birmingham, School of Biosciences, Institute of Microbiology and Infection, United Kingdom
[4]Current address: Broad Institute of MIT and Harvard, Cambridge, MA 02142.

The GIL01 bacteriophage, is a temperate phage, which infects the insect pathogen *Bacillus thuringiensis*. During the lytic cycle, phage gene transcription is initiated from three promoters: *P1* and *P2*, which control the expression of the early phage genes involved in genome replication and *P3*, which controls the expression of the late genes responsible for virion maturation and host lysis. Unlike most temperate phages, GIL01 lysogeny is not maintained by a dedicated phage repressor but rather by the host's regulator of the SOS response, LexA. Previously we showed that the lytic cycle was induced by DNA damage and that LexA, in conjunction with phage-encoded protein gp7, repressed *P1*. Here we examine the lytic/ lysogenic switch in more detail and show that *P3* is also repressed by a LexA/ gp7 complex, binding to tandem LexA boxes within the promoter. We also demonstrate that expression from *P3* is considerably delayed after DNA damage, requiring the phage-encoded DNA binding protein, gp6. Surprisingly, gp6 is homologous to LexA itself and, thus, is a rare example of a LexA homologue directly activating transcription. We propose that the interplay between these two LexA family members, with opposing functions, ensures the timely expression of GIL01 phage late genes.

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CRISPR/Cas9-MEDIATED GENOME EDITING IN CABBAGE

Ester Stajič[1], Jana Murovec[1], Borut Bohanec[1]

[1]University of Ljubljana, Biotechnical Faculty, Department of Agronomy, Slovenia

Induction of haploids is an important technique for production of homozygous lines which can be later crossed to obtain hybrids with high agronomic value. One of the possible techniques for production of parental homozygous plants is also pollination with inducer lines. Different point mutations in the centromere-specific histone H3 variant (CENH3) have been published that lead to a haploid offspring after crossing with wild-type plants which suggests that plants carrying those mutations could be used as haploid inducers (Karimi-Ashtiyani et al., 2015). Due to great importance of hybrids in plant breeding, CENH3 protein was chosen as a target for CRISPR/Cas9 protocol development.

In our first experiments, a protocol for protoplast isolation was developed. Several parameters affecting the efficiency of protoplast isolation were tested and protoplasts with high viability were isolated using overnight incubation in enzyme solution from leaves of *in vitro* grown plants. Later, vectors for editing two different sites in *cenh3* in cabbage were prepared. For each site, three sgRNAs were designed. To induce nucleotide substitution by homology-mediated repair pathway a repair template was also cloned into plasmids. Prepared vectors (with or without repair template) were tested using protoplast transformation and infiltration of *Agrobacterium tumefaciens* into cabbage leaves. Next-generation sequencing (NGS) was used for detection of induced specific mutations. For regeneration of mutagenized plants, two different protocols were optimized: *Agrobacterium tumefaciens* mediated transformation and regeneration of protoplasts.

In the future, we plan to obtain plants carrying desired mutations in CENH3 through delivery of our cloned plasmids into cabbage cells by stable transformation and to use the developed protocol for genome editing of other targets.

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FATTY ACID ABNORMALITIES IN IBD ARE LINKED TO NOVEL IBD SPECIFIC LOCI

G. Jezernik[1], U. Potočnik[1,2]

[1]University of Maribor, Faculty of Medicine, Slovenia
[2]University of Maribor, Faculty of Chemistry and Chemical Engineering, Slovenia

Introduction: Inflammatory bowel disease (IBD) is a common immune-mediated complex disease characterized by chronic inflammation of the gastrointestinal tract. Current understanding of IBD pathogenesis highlights inappropriate and inadequate immune responses to gut microbes which arise in a genetically susceptible host. Fatty acid profile abnormalities have been associated with IBD independent of nutritional status or disease activity. However, no study so far has attempted to look for overlap between IBD and fatty acid associated loci or investigate the genetics of fatty acid profiles in IBD. To this end, we conducted a comprehensive genetic study of fatty acid profiles in IBD.
Materials and methods: Peripheral blood samples from 55 controls and 77 IBD patients were fractionated to lymphocyte and erythrocyte fractions. DNA was isolated from lymphocyte fraction. Genotyping was performed using iCHIP, a custom microarray platform designed for fine mapping of susceptibility loci in immune-related diseases. Lipids in erythrocyte fraction were processed and analyzed using gas chromatography with flame ionization detector to obtain fatty acid profile data. Genotype and fatty acid profile data was analyzed using R, PLINK and SPSS software packages. In addition, the e1071 R package was used for construction of bootstrap aggregated support vector machine (SVM) classifiers.
Results: We identified 6 loci (near *CBS*, *LRRK2*, *FRMPD4*, *TMCOA5*, *ZNF767P* and *ABCA12*) significantly associated with fatty acids specifically in IBD. Most significant loci is located near the *CBS* gene ($p = 7.62 \times 10^{-8}$). Of note, we also replicated the fatty acid associated FADS gene locus. We also replicated previously observed changes in fatty acid profiles in IBD and the observed inverse correlation between oleic and arachidonic acid levels. In addition, using genetic and fatty acid data, it is possible to construct SVM classifiers with 100% accuracy. However, independent validation in a larger cohort is required to confirm the viability of such classifier for potential diagnostic purposes.
Conclusions: Our results highlight the involvement of fatty acids and fatty acid associated loci in IBD and encourage further investigations of the genetics of fatty acid profile changes in IBD and the role of fatty acids in IBD pathogenesis.

THE INVISIBILITY CLOAK: CHITIN BINDING PROTEIN OF *Verticillium nonalfalfae* DISGUISES FUNGUS FROM PLANT CHITINASES

Helena Volk[1], Kristina Marton[1], Marko Flajsman[1], Sebastjan Radisek[2], Ingo Hein[3], Crtomir Podlipnik[4], Branka Javornik[1], Sabina Berne[1]

[1]Biotechnical Faculty, Department of Agronomy, Slovenia
[2]Slovenian Institute of Hop Research and Brewing, Department of Plant Protection, Slovenia
[3]The James Hutton Institute, Cell and Molecular Sciences, United Kingdom
[4]Faculty of Chemistry and Chemical Technology, Department of Chemistry and Biochemistry, Slovenia

Verticillium nonalfalfae (*Vna*) is a phytopathogenic ascomycete that infects various important crops, including hops. The plant immune system responds to fungal infections when the extracellular lysin motifs (LysM) of plant immune receptors recognize GlcNAc, the structural component of the fungal chitin cell wall. We report here that *Vna* outsmarts this recognition process by secreting a chitin binding protein (CBP) that disguises the fungus from the plant immune system.

The highest expression of *Vna8.213*, the gene encoding for CBP, was detected in stems of susceptible hop at the latest stages of infection. Examination of the sequence variation of *Vna8.213* in different *Vna* and *V. alfalfae* (*V*a) isolates, revealed no polymorphisms among *Vna* or *Va* isolates, while *Vna* and *Va* sequences share 97% identity on the nucleotide level, suggesting that this gene is highly conserved and important for the fungal lifestyle.

Monitoring plant immune responses to recombinant CBP demonstrated that CBP is not recognised by the plant immune system, since no hypersensitive response was observed after infiltration in *Nicotiana benthamiana* leaves and there was no extracellular medium alkalinisation or ROS generation in the tobacco BY-2 and *Arabidopsis* T87 cell lines. The nonspecific subcellular localisation of *in planta* transiently expressed CBP::mRFP fusion additionally confirms these findings.

A carbohydrate sedimentation test showed specific binding of the recombinant CBP to chitin. The specificity of the interaction was confirmed by surface plasmon resonance (Kd: 0.78 ± 0.58 μM) and 3D modelling of the interaction between $(GlcNAc)_7$ and CBP was performed in Yasara, to determine the interacting residues. In a cell protection assay, 3 μM recombinant CBP caused hyphae aggregation and shielded *Trihoderma viride* from degradation by xylem sap chitinases.

This study revealed one of the secrets of the successful lifestyle of *Vna:* the secreted CBP acts as a fungal invisibility cloak. CBP, by binding to the chitin cell wall, acts as a shield against xylem sap chitinases and interferes with the plant immune system by outcompeting plant LysM receptors.

TRANSCRIPTOME ANALYSIS AND CELLULAR BACKGROUND OF THE PIGMENT PATTERN IN MARBLE AND BROWN TROUT

Ida Djurdjevič[1], Mateja Erdani Kreft[2], Tomasz Furmanek[3], Seita Miyazawa[4], Simona Sušnik Bajec[1]

[1]University of Ljubljana, Biotechnical Faculty, Department of Animal Science, Slovenia
[2]University of Ljubljana, Faculty of Medicine, Institute of Cell Biology, Slovenia
[3]University of Bergen, Department of Informatics, Computational biology unit, Norway
[4]Osaka University, Graduate School of Frontier Biosciences, Japan

Variety of color and pigment patterns present in the animal kingdom has always fascinated researchers. Fishes are known to be particularly rich in colors and pigment patterns. In our study, we searched for cellular and genetic mechanisms responsible for the arrangement of pigment cells into a unique labyrinth pattern in marble trout (*Salmo marmoratus*) skin in contrast to a more common spot pattern in the skin of a closely related brown trout (*S. trutta*).
Pigment cells ultrastructure and their position in the skin were examined with transmission electron microscopy. The position of pigment cells in the skin of brown trout was more organized, compared to that of marble trout. In addition, erythrophores were found exclusively in the brown trout skin. To investigate cell-cell interactions, potentially influencing skin pigment pattern, we prepared and observed a primary pigment cell culture from the skin of both species. Based on these results, a clear connection between the presence, position and interaction of specific pigment cell type and different pigment pattern in the skin could not be proposed.
Transcriptomes (RNA-seq) of cells from differently pigmented regions were sequenced in order to find candidate genes for trout pigment pattern. A large set of differently expressed transcripts between species, but also between differently pigmented regions, were found. Among these transcripts, there were several coding for membrane proteins, which are involved in cell-cell communication. We tested 22 of candidate genes with real time PCR on more biological replicates per species. The expression of six of these genes was tested in hybrids between the two species. The genes *p21, gja5* and *tjp1* were displaying the highest correlation between their expression level and skin pattern. Genes *gja5* and *tjp1* code for proteins present in different cell junctions and can potentially affect the cell-cell communication; genes *p21* and *tjp1* can affect actin stress fibre formation and regulation of cell migration; gene *p21* is also differentially expressed in different melanocytes in human. We propose hypotheses about the function of these genes in the process of different pigment pattern formation in trout.
Based on our results, it can be concluded that specific i) communication between pigment cells and ii) cytoskeletal rearrangements are responsible for the pigment pattern formation in trout.

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THE *ESCHERICHIA COLI* COLIBACTIN RESISTENCE PROTEIN ClbS IS A NOVEL DNA BINDING PROTEIN THAT PROTECTS DNA FROM NUCLEOLYTIC DEGRADATION

Molan Katja[1], Podlesek Zdravko[1], Butala Matej[1], Hodnik Vesna[1], Žgur Bertok Darja[1]

[1]University of Ljubljana, Biotechnical Faculty, Department of Biology, Slovenia

The gut microbiota plays a significant role in human health and development. On the other hand, to survive and reproduce, pathogenic microorganisms provoke specific responses and damage within the host. The *clbS* gene is located in the genomic island *pks* that encodes the production of colibactin, a potent genotoxin. The *pks* island is found among the *Enterobacteriacea,* in particular *E. coli* strains of the B2 phylogenetic group and *Klebsiella pneumoniae*. Colibactin causes chromosomal instability as well as DNA damage and has been associated with colorectal cancer. The prevalence of *E. coli* isolates from the B2 phylogenetic group has been increasing significantly in developed countries. The genotoxin producer must be protected against its own toxin and recent investigations have shown that ClbS provides protection against colibactin. As members of the *E. coli* B2 phylogenetic group encode other genotoxins, including the uropathogenic specific protein Usp, we postulated that ClbS might protect genome integrity by directly interacting with the nucleoid. Here we show that the *clbS* gene product enables growth of an *E. coli usp*$^{+}$ strain lacking its cognate immunity proteins, Imu1-3. Further, based on expression of the DNA damage reporter *recA-gfp* gene fusion, we showed that ClbS decreases Usp-induced DNA damage. This was confirmed *in vitro* by showing that the ClbS protein protects DNA against several DNases. To get insight into the ClbS DNA-protective mechanism, we investigated the DNA binding ability of the ClbS protein using two approaches: electrophoretic shift assay and surface plasmon resonance spectrometry. Both methods confirmed that ClbS non-specifically interacts with DNA, exhibiting higher affinity for single-stranded DNA that can be exposed at sites of DNA damage. Further, ClbS tryptophan fluorescence emission spectra in the DNA bound or unbound state, imply that ClbS forms DNA aggregates.

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PERITUMORAL GENE ELECTROTRANSFER OF INTERLEUKIN-12 AS IMMUNE BOOST TO INTRATUMORAL ELECTROCHEMOTHERAPY FOR TREATING MURINE MELANOMA

Katja Uršič[1], Špela Kos[1], Urška Kamenšek[1], Maja Čemažar[1,2], Gregor Serša[1,3]

[1]Institute of Oncology Ljubljana, Department of Experimental Oncology, Slovenia
[2]University of Primorska, Faculty of Health Sciences, Slovenia
[3]University of Ljubljana, Faculty of Health Sciences, Slovenia

Treatment of metastatic melanoma has undergone rapid renovation, especially in the light of combining established therapies with recently discovered immunotherapies. Our research group has been studying electrochemotherapy, which is a well-established local ablative therapy in clinics across Europe. Although local effectiveness of electrochemotherapy is up to 80% of tumor control, no noticeable effects on distant non-treated metastases (abscopal effect) have been observed[1]. To achieve both, better local tumor growth control and an abscopal effect, we combined equally effective electrochemotherapy using immunomodulatory chemotheotherapeutics (cisplatin, oxaliplatin and bleomycin) with peritumoral gene electrotransfer of interleukin-12 (IL-12) as an adjuvant immunotherapy.[2] The combination therapy was tested using B16F10 malignant melanoma primary tumor model and two dual-flank melanoma models mimicking systemic disease. Tumor growth was followed and tumor and blood samples were collected for immunohistochemical analysis of the tumor microenvironment and for the detection of IL-12 expression. With peritumoral gene electrotransfer of therapeutic plasmid encoding mouse IL-12 under constitutive EF-1α/HTLV hybrid promoter, we achieved low dose expression of the transgene in surrounded skin tissue, without any systemic cytotoxicity. In comparison to either monotherapy which resulted only in a delayed growth of treated tumors, with the novel combination therapy up to 38 % of mice were cured. After the combination therapy, consisting of cisplatin in electrochemotherapy with an IL-12 boost, a delayed tumor growth of the distant non-treated tumor was observed. We showed that gene electrotransfer of IL-12 potentiates the effect of electrochemotherapy on local and systemic level. Our findings indicate a potential clinical application of the combination therapy for malignant melanoma treatment.

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POTENTIAL AVIRULENCE GENE *Vna8.691* OF PHYTOPATHOGENIC FUNGUS *Verticillium nonalfalfae*

Kristina Marton[1], Sebastjan Radišek[2], Marinka Horvat[1], Helena Volk[1], Jernej Jakše[1], Branka Javornik[1], Sabina Berne[1]

[1]Biotechnical Faculty, Department of Agronomy, Slovenia
[2]Slovenian Institute of Hop Research and Brewing, Department of Plant Protection, Slovenia

Verticillium nonalfalfae (*Vna*) is a phytopathogenic ascomycete, which causes Verticillium wilt of various plants. Fungal effectors are key players in disease development and progression, since they either modulate host immune responses (virulence factors) or trigger them after being recognized by different plant receptors (avirulence factors). Recently, several *Vna* candidate effectors were identified during infection of hops (*Humulus lupulus* L.) by custom-made bioinformatics pipeline and their implication in disease was determined by pathogenicity testing of fungal deletion mutants (Marton et al., 2017). Here, we focus on characterization of one particular effector gene *Vna8.691*, whose deletion mutants showed increased virulence after infection of susceptible hop cultivar 'Celeia', suggesting it could be an avirulence gene (Houterman et al., 2009; de Jonge et al., 2012).
It encodes a small putatively secreted neutral protein of 9.9 kDa with the highest similarity to protein BN1708_009912 from *V. longisporum*. It is a typical fungal effector as predicted by EffectorP, without known protein domains or motifs according to amino acid sequence analysis with blast or HMMER searches against NCBI, PFAM, InterPro, CATH and OrthoDB databases. Preliminary subcellular localization studies using fluorescence microscopy revealed nucleo-cytoplasmic localization of transiently expressed mRFP-tagged Vna8.691 after agroinfiltration in *Nicotiana benthamiana* leaves. Gene expression profiling by qRT-PCR indicated *Vna8.691* is highly expressed in the roots of susceptible hop at early stages of infection (6 dpi), while its expression drastically declines at the onset of disease symptoms. In the shoots of susceptible hop, expression of *Vna8.691* is almost 4 fold lower and declining with time course of disease. The *Vna8.691* expression in resistant 'Wye Target' is moderate in roots and barely detected in shoots. PCR-amplification of *Vna8.691* in infected plant samples confirmed its presence in all *V. nonalfalfae*, *V. alfalfae* and *V. longisporum* isolates, while it was discovered in one third of *V. dahliae* and in one half of *V. isacii* and *V. albo-atrum* isolates.
In order to implicate *Vna8.691* as avirulence gene, we plan pathogenicity testing of Δ*Vna8.691* deletion mutant in resistant hop and expect the development of more severe symptoms compared to wild type fungus. In addition, yeast two-hybrid (Y2H) screening of cDNA library from infected hop might reveal cognate R protein responsible for recognition of this putative Avr effector, which could serve in hop resistance breeding to Verticillium wilt.

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GENETIC, BIOFILM AND CELLULAR ANALYSIS OF UROPATHOGENIC VIRULENCE FACTORS OF VARIOUS HUMAN *ESCHERICHIA COLI* STRAINS EMPLOYING A BIOMIMETIC UROTHELIAL MODEL *IN VITRO*

Luka Predojević[1], Darja Žgur-Bertok[1], Darja Keše[2], Mateja Erdani Kreft[3], Marjanca Starčič Erjavec[1]

[1] University of Ljubljana, Biotechnical Faculty, Department of Biology, Slovenia
[2] University of Ljubljana, Faculty of Medicine, Institute of Microbiology and Immunology, Slovenia
[3] University of Ljubljana, Faculty of Medicine, Institute of Cell Biology, Slovenia

Bacterium *Escherichia coli* (*E. coli*) is a part of normal gut microbiota. Certain strains, however, can cause different infections, among them also urinary tract infections (UTI). *E. coli* strains causing UTI are designated as uropathogenic strains of *E. coli* (UPEC). Research of UPEC strains uropathogenicity is a fast growing field and many different models are already established with their own advantages and limitations. Our goal was to find a correlation between pathogenicity of different human *E. coli* strains, their genetic background and ability to form biofilm employing our biomimetic urothelial model *in vitro* derived from porcine urinary bladder urothelium.

In our study we used 12 uropathogenic (UPEC) strains, 11 fecal *E. coli* strains (FEC) isolated from healthy humans as well as two laboratory *E. coli* strains MG1655 and DH5α.

Methods included viability tests of the urothelial cells in the *in vitro model* after *E. coli* infection, biofilm assays and PCR for detection of selected virulence factor genes of among studied.

In general, results of the urothelial cells' viability in the used biomimetic urothelial model *in vitro* corresponded with the type of the *E. coli* strain. The viability of the cells in our model, when infected by UPEC strains was much lower than the viability of the cells after infection with FEC strains. The total number of virulence factor genes did not correlate with the viability level, but certain genes (*cnf1*, *hlyA*, *clbA*Q, *papGIII*, *sfaDE* and *tcpC*) were more frequently found among strains, which caused a significant lower viability of urothelial cells. The biofilm assays (formed biofilm measured as absorbance at 570 nm) showed no correlation between certain group of strains (either FEC or UPEC) or level of viability (high or low) and the amount of formed biofilm.

In conclusion, genetic, biofilm and cellular analysis of human UPEC and FEC strains showed that among UPEC, as well as FEC strains, some strains possessed more virulence factor genes, were forming more biofilm and were causing lower urothelial cells viability in the *in vitro* model, however there was no correlation between biofilm formation, number of virulence factor gene and low viability.

GENETIC VARIABILITY IN OXIDATIVE STRESS PATHWAYS INFLUENCES THE OCCURRENCE OF ADVERSE EVENTS OF DOPAMINERGIC TREATMENT IN PARKINSON'S DISEASE

Sara Redenšek[1], Maja Trošt[2], Vita Dolžan[1]

[1]University of Ljubljana, Faculty of Medicine, Institute of Biochemistry, Pharmacogenetics Laboratory, Slovenia
[2]University Medical Centre Ljubljana, Department of Neurology, Slovenia

Introduction: Oxidative stress is one of the pivotal underlying processes driving Parkinson's disease' (PD) pathogenesis. Elevated biomarkers of oxidative stress in body fluids of PD patients and increased activities of ROS scavenging enzymes' were reported. Candidate-gene and GWAS studies also showed associations of genetic variability in these pathways with PD risk. However, genetic variability in oxidative stress pathways has not been studied in association with PD treatment outcome yet. Our aim was to investigate if selected single nucleotide polymorphisms (SNPs) in oxidative stress pathways are associated with adverse events (AEs) of dopaminergic treatment in PD.
Materials and methods: We enrolled 224 PD patients and collected their demographic and clinical data including AEs (motor fluctuations, dyskinesia, nausea/vomiting, orthostatic hypotension, peripheral oedema, excessive daytime sleepiness (EDS) and sleep attacks, visual hallucinations, and impulse control disorders (ICDs)). DNA was isolated from peripheral blood samples and genotyped for *NOS1* rs2293054, *NOS1* rs2682826, *CAT* rs10836235, *CAT* rs1001179, and *SOD2* rs4880. Statistical analysis using logistic regression was performed.
Results: The study included 57.6% males and 42.4% females, with median age at onset 62.2 (55.0-71.6) years, and median disease duration 7.6 (3.8-14.0) years. Levodopa equivalent dose at enrolment was 975 (600-1348) mg/day, while median levodopa treatment duration was 6.3 (2.5-11.1) years. *NOS1* rs2682826 A allele was associated with increased odds for EDS and sleep attacks (OR=1.75; 95%CI=1.00-3.06, p=0.048), *SOD2* rs4880 T allele was associated with lower odds for nausea/vomiting (OR=0.49, 95%CI=0.25-0.94; p=0.031), *CAT* rs1001179 A allele was associated with higher odds for peripheral oedema development (OR=3.10; 95%CI=1.48-6.52; p=0.003), and finally *NOS1* rs2682826 A allele was also associated with greater odds for ICDs (OR=2.59; 95%CI=1.09-6.19; p=0.032). All associations were adjusted for significant clinical parameters. We did not find any associations between selected SNPs and motor AEs.
Conslucions: Our study suggests a role of oxidative stress pathways in non-motor AEs of dopaminergic treatment in PD. Further studies are needed to establish these genetic factors as biomarkers for personalizing PD treatment.

Loree Heller, Old Dominion University, Frank Reidy Research Center for Bioelectrics and Old Dominion University, School of Medical Diagnostic and Translational Sciences, Norfolk, USA

Cytosolic Pattern Recognition Receptors are activated by DNA Electroporation

Michael Kabesch, University Children's Hospital Regensburg (KUNO), Department of Pediatric Pneumology and Allergy, Regensburg, Germany

Genetics and Epigenetics of Asthma and Asthma Therapy

Maja Čemažar, Institute of Oncology Ljubljana, and University of Primorska, Faculty of Health Sciences, Slovenia

Ablative Therapies Combined with Interleukin-12 Gene Electrotransfer for Treatment of Tumors of Client-Owned Dogs

Richard Heller, Old Dominion University, Frank Reidy Research Center for Bioelectrics and Old Dominion University Institution, School of Medical Diagnostics and Translational Research, USA

Potential Therapeutic Applications Using Gene Electrotransfer

David Dobnik, National Institute of Biology, Department of Biotechnology and Systems Biology, Slovenia

Characterization of Viral Vectors for Gene Therapy: Molecular Biology Hand-in-Hand with Electron Microscopy

Boštjan Markelc, CRUK/MRC Oxford Institute for Radiation Oncology, Department of Oncology, University of Oxford, UK and Department of Experimental Oncology, Institute of Oncology Ljubljana, Slovenia

Tumour Endothelium, Sprouting Angiogenesis and Their Response to Radiotherapy


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CYTOSOLIC PATTERN RECOGNITION RECEPTORS ARE ACTIVATED BY DNA ELECTROPORATION

Loree Heller[1,2], Nina Semenova[1] , Katarina Znidar[3] , Masa Bosnjak[4] , Maja Cemazar[3,4]

[1]Old Dominion University, Frank Reidy Research Center for Bioelectrics, USA
[2]Old Dominion University, School of Medical Diagnostic and Translational Sciences, Norfolk, USA
[3]University of Primorska, Faculty of Health Sciences, Slovenia
[4]Institute of Oncology Ljubljana, Department of Experimental Oncology, Slovenia

The application of controlled electric pulses (electroporation), enhances delivery and expression of plasmid DNA (pDNA) to a wide variety of healthy tissues as well as many tumor types. Electroporation of pDNA encoding therapeutic genes has reached veterinary and human clinical trials for cancer therapies, cancer vaccines, and infectious disease vaccines. It is well-established that in vivo DNA delivery by electroporation and by other physical and chemical methods is accompanied by inflammatory effects, which may modulate the efficacy of any non-viral gene therapy. The root cause of these inflammatory effects may be the activation of pattern recognition receptors (PRRs). These receptors are found in all mammalian cells and represent an ancient, germlineencoded, immune mechanism to detect molecules typical of pathogen invasion. Receptor activation is also associated with the aberrant presence of genomic DNA in the cytosol found in autoimmune diseases. Binding and activation of these primitive innate immune sensors induces the production of proinflammatory proteins and diverse forms of programmed cell death. Along with the well-known endosomal PRR TLR9, more than 15 cytosolic DNA-specific PRRs have been described. However, the detailed mechanisms of nucleic acid sensing have not been elucidated. After intracellular delivery, we hypothesize that pDNA may also bind and activate intracellular DNA-specific PRRs. Our studies demonstrate that both normal and cancer cells in suspension or in 3D culture respond similarly to pDNA electroporation with the production of the inflammatory markers IFNβ and TNFα and the induction of cell-type dependent cell death. In addition, significant upregulation of the mRNAs and proteins of several PRRs, most commonly DAI/ZBP1, DDX60, and IFI16/p204, is observed after pDNA delivery. To support the hypothesis, we recently demonstrated direct binding of pDNA by DAI/ZBP1 but not by other PRRs within minutes of pDNA electroporation. These initial observations support the concept that DNA-specfic PRRS are activated by DNA electroporation to induce inflammation and cell death. Better understanding of the molecular underpinnings of nucleic acid sensing by PRRs may allow us to modulate these possibly unavoidable effects of non-viral DNA delivery.

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GENETICS AND EPIGENETICS OF ASTHMA AND ASTHMA THERAPY

Michael Kabesch[1]

[1]University Children's Hospital Regensburg (KUNO), Department of Pediatric Pneumology and Allergy, Regensburg, Germany

Asthma is a chronic inflammatory disease of all ages affecting approximately 300 million people worldwide (1). Genetic susceptibility and environmental exposures contribute to the disease and interact in the often early onset and maintenance of asthma (2). Genome wide association studies identified robust genetic susceptibility markers as early as 2007, when we first published a GWAS on childhood asthma within our GABRIEL consortium (3). Later we could show that genetic susceptibility for childhood onset asthma and adult onset asthma differs (4) and that gender specific effects can be observed (5). Recently, the GABRIEL consortium published the last great GWAS on asthma with more than 100.000 cases included worldwide (6). Furthermore, major genetic susceptibility factors such as those from chromosome 17q21 interact with environmental stimuli like cigarette smoke exposure (7) and protective effects of early farm live (8), pointing towards gene by environment and gene by microbiome interactions.

One possible mechanism by which such interactions may occur is epigenetic programming (9). Indeed, farm exposure as well as the development of asthma lead to epigenetic signatures such as differences in methylation throughout the genome (10). In a recent hallmark study (11), the genome wide effect of fetal smoke exposure during pregnancy was characterized using massive pyrosequencing of mothers and offspring over different time points.

While these studies and results increased our knowledge and understanding of the disease, we are still fare away from being able to use this information in clinical practice in the context of personalized or stratified medicine. There, we face the challenge of asthma patients that do not respond to standard steroid therapy experiencing frequent and severe exacerbations. It is expected that the combination of different omics technologies will identify mechanisms in of therapy refractory (12). Only recently, biologicals such anti-IgE, anti IL-5 and anti IL-4/IL-13 have been introduced to the asthma field and it will be a future challenge to characterize patients on the individual molecular level that well respond to these tailored but also very expensive therapies. First steps in this direction are currently ongoing.

ABLATIVE THERAPIES COMBINED WITH INTERLEUKIN-12 GENE ELECTROTRANSFER FOR TREATMENT OF TUMORS OF CLIENT-OWNED DOGS

Maja Čemažar[1,2]

[1]Institute of Oncology Ljubljana, Slovenia
[2]University of Primorska, Faculty of Health Sciences, Slovenia

Ablative therapies for cancer include standard therapies such as surgery and radiotherapy and novel, such as electrochemotherapy. Electrochemotherapy is a local ablative technique where cytotoxicity of chemotherapeutic drugs is increased by the use of applied electric pulses (electroporation) to the tumors [1]. It is used for treatment of various types of tumors in human and veterinary oncology in Europe. However, electrochemotherapy is a local treatment therefore, it has no or minor effect on distant tumors or metastases. In the case of radiotherapy, sporadically, bystander and abscopal effect was observed. Therefore, to boost and enhance the systemic antitumor effect of local ablative therapies, the combination of these therapies with different immune therapies was proposed[4]. Among different immune therapies, gene electrotransfer of IL-12 showed great promise in preclinical studies and in studies treating human and veterinary patients[5,6]. In our preclinical studies on different murine tumors, combination of electrochemotherapy or radiotherapy with IL-12 gene therapies resulted in different levels of complete responses depending on the tumor model, route of administration of plasmid encoding IL-12 and timing of the combined therapy. In addition, antibiotic resistance free plasmid encoding IL-12 under inducible and tissue types specific promoters were also evaluated in preclinical studies. In veterinary oncology, electrochemotherapy combined with peritumoral human interleukin-12 (hIL-12) gene electrotransfer was used for treatment of mast cell tumors in 18 client-owned dogs. Local tumor control, recurrence rate, as well as safety of combined therapy were evaluated. One month after the therapy, no side effects were recorded and good local tumor control was observed with high complete responses rate, which even increased during the observation period to 72%. IL-12 gene electrotransfer resulted in 78% of patients with detectable serum IFN-? and/or IL-12 levels. In the treated tumors vascular changes as well as minimal T-lymphocytes infiltration was observed. After one week, the plasmid DNA was not detected intra- or peritumorally and no horizontal gene transfer was observed[7,8]. To further improve the therapy, a plasmid encoding canine IL-12 (caIL-12) was constructed and in our on-going clinical trial 56 client-owned dogs of different breeds, whose owners refused any other type of standard treatment were included so far. The updated results of the current study will be presented.

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POTENTIAL THERAPEUTIC APPLICATIONS USING GENE ELECTROTRANSFER

Richard Heller[1,2], Chelsea Edelblute[1] , Cathryn Lundberg[1] , Sezgi Arpag-McIntosh[1] and Anna Bulysheva[1]

[1]Old Dominion University, Frank Reidy Research Center for Bioelectrics, United States
[2]Old Dominion University Institution, School of Medical Diagnostics and Translational Research

United States Gene therapy has held great promise for effective treatment of a variety of disorders including cancer, metabolic disorders, cardiovascular diseases and genetic disorders as well as a prophylactic approach for infectious diseases and cancer. Although Gene therapy has made great strides during the past decade, there have been setbacks that include toxicity (e.g. adenoviral immunogenicity and retroviral insertional mutagenesis) or efficacy (e.g. low expression levels with non-viral delivery). It is clear that a significant effort is still needed and that delivery, particularly delivery to a specific target,still remains an important hurdle. One area that has seen tremendous growth is non-viral gene delivery systems including the use of physical delivery approaches. Of the physical delivery methods, electroporation (gene electrotransfer; (GET)) has made the most progress with over 80 clinical trials. GET has been accepted as a viable approach to achieve effective delivery and has been tested in a variety of tissues. Expression of the transgene can be controlled by manipulating the GET parameters such as pulse width, applied voltage, pulse number and electrode configuration. This control of the expression profile is a key element in developing successful therapeutic applications. We have utilized GET for delivering DNA vaccines as well as to evaluate the potential for accelerating wound healing or for inducing reperfusion of ischemic tissue. Work has also been focused on developing an immune gene transfer approach for the treatment of cancer. Delivery of plasmids encoding cytokines directly to tumors has been shown by our lab and others to induce not only a local immune response, but a systemic one as well. The positive responses were directly related to the ability to achieve the appropriate expression profile following delivery of the plasmid. This approach has been successfully tested in multiple Phase II clinical trials. GET is an effective approach for delivering plasmid DNA and the number of applications has been steadily growing.

CHARACTERIZATION OF VIRAL VECTORS FOR GENE THERAPY: MOLECULAR BIOLOGY HAND-IN-HAND WITH ELECTRON MICROSCOPY

David Dobnik[1*], Polona Kogovšek[1], Tjaša Jakomin[1], Nejc Košir[1], Magda Tušek Žnidarič[1], Denis Kutnjak[1], Maja Ravnikar[1]

[1]National Institute of Biology, Department of Biotechnology and Systems Biology, Slovenia

Viral vectors have emerged as safe and effective delivery vehicles for clinical gene therapy. Manufacturing of clinical grade vectors requires knowledge of the complex methods needed to generate, purify and characterize viral vectors. One of the most crucial, but also problematic steps, is the characterization of viral vectors in different production phases during process development and in final drug substance. Our test subject was one of the most commonly used viruses for gene therapy, adeno-associated virus (AAV), which we used to evaluate congruency analytical results obtained with different approaches. As representatives of molecular methods, which provide the data about the viral genome quantity in the sample, we used quantitative real-time PCR (qPCR) and digital PCR (dPCR), which enable relative and absolute quantification of target nucleic acids, respectively. Both techniques were compared side by side on a set of assays targeting four genes of the viral genome construct. Results of the analyses showed that dPCR performs better in terms of accuracy and variability (average coefficient of variability <10 %). Moreover, we have shown that pre-treatment of samples prior to PCR analysis is also important. As the presence of viral genomes does not give any indication on viral capsid integrity, we complemented molecular methods with transmission electron microscopy (TEM) for direct observation of viral particles. With TEM analysis we evaluated viral structure, presence of full and empty/damaged/broken capsids and presence of impurities. We were also able to confirm the differences in viral titers observed by qPCR and dPCR determined after different pre-treatments, by connecting those results to the initial sample integrity determined by TEM. Finally, to characterize viral construct and detect the presence of other nucleic acids in the sample, we introduced Oxford Nanopore MinION sequencing. Using nanopore sequencing, we confirmed the sequence of the construct and were also able to identify some impurities present in the samples of viral vectors (e.g. remaining of plasmids). To conclude, we showed that for the most informative characterization of viral vectors it is crucial to use a combination of molecular approaches and microscopy that characterize viral vectors from different perspectives. Relatively new technologies offer additional information, which nicely complements more traditional analytical approaches.

TUMOUR ENDOTHELIUM, SPROUTING ANGIOGENESIS AND THEIR RESPONSE TO RADIOTHERAPY

Boštjan Markelc[1, 2] Jakob Kaeppler[1], Mario Buono[3], Russell Bates[4], John Prentice[1], James Thompson[1], Mark Hill[1], Mike Brady[1, 5], Julia Schnabel[6], Ruth Muschel[1]

[1]CRUK/MRC Oxford Institute for Radiation Oncology, Department of Oncology, University of Oxford, UK
[2]Department of Experimental Oncology, Institute of Oncology Ljubljana, Slovenia
[3]MRC Molecular Hematology Unit, University of Oxford, UK
[4]Oxford Institute of Biomedical Engineering, Department of Engineering Science, University of Oxford, UK
[5]Department of Oncology, University of Oxford, UK
[6]Biomedical Engineering Department, King's College London, UK

The aberrant tumour vasculature is a crucial part of the tumour microenvironment, contributing to tumour growth and response to treatment e.g. radiotherapy. Radiotherapy affects not only cancer cells, but also the tumour microenvironment, including endothelial cells. However, their response to radiotherapy including is incompletely understood. Our aim was to investigate the biological underpinnings of tumour blood vessel response to radiotherapy and its impact on vessel structure and function.
Intravital microscopy combined with transgenic mice with a VECadherin-Cre-ERT2 construct that in combination with a loxP-flanked STOP tdTomato cassette results in the expression of fluorescent proteins in endothelial cells was used. An abdominal window chamber was surgically implanted and GFP labelled tumours were induced. Upon imaging, a blood pool labelling agent was injected. This setting enabled us to follow tumour growth, blood vessel development and perfusion within the same tumour over time. We followed the response of tumour vasculature to single high dose and fractionated radiotherapy by intravital microscopy. Obtained 3D microscopy images of the whole tumour, its vasculature and perfusion were analysed, using a machine learning algorithm. Finally, markers for proliferation and apoptosis in tumour endothelial cells were determined after irradiation, as well as changes in mRNA expression of endothelial cells isolated from irradiated tumours, were investigated by RNA sequencing.
Intravital microscopy revealed distinct patterns of global tumour vasculature development. We visualized and quantified dynamic sprouting angiogenesis in tumours and follow tumour vasculature development and function over a 12 days' period within the same tumour. When tumours were irradiated, the development of tumour vasculature was arrested, resulting in a decrease in the number of newly formed sprouts. Additionally, we saw an increase of the diameter/length ratio of established sprouts, improved perfusion of remaining tumour vessels and an increase of directional coherence. This was accompanied with an arrest of endothelial cell proliferation and a modest increase in endothelial cell apoptosis accompanied by downregulation of angiogenesis markers.
The developed transgenic mouse model in combination with intravital imaging approach and in-house image analysis increases our understanding of the tumour vasculature and its response to radiotherapy.


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Ellen Zechner, Institute of Molecular Biosciences, University of Graz, BioTechMed-Graz, Austria

Molecular Functions of Microbiota-Derived Enterotoxins in Human Intestinal Disease

Marjanca Starčič Erjavec, University of Ljubljana, Biotechnical Faculty, Department of Biology, Ljubljana, Slovenia

Construction and Antibacterial Effect of the Strain ŽP – the First Bacterial Conjugation-Based "Kill"–"Anti-Kill" Antimicrobial System

Tomaž Accetto, University of Ljubljana, Biotechnical faculty, Animal Science department, Slovenia

The Lytic *Myoviridae* of *Enterobacteriaceae* form Tight Recombining Assemblages Separated by Discontinuities in Genome Average Nucleotide Identity and Lateral Gene Flow

Ivan Toplak, University of Ljubljana, Veterinary Faculty, Institute of Microbiology and Parasitology, Slovenia

Identification and Characterization of Pathogens of Infectious Disease with the Method of Next Generation Sequencing


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MOLECULAR FUNCTIONS OF MICROBIOTA-DERIVED ENTEROTOXINS IN HUMAN INTESTINAL DISEASE

K. Unterhauser, L. Pöltl[‡], G. Schneditz[‡], S. Kienesberger, R. A. Glabonjat[2], J. Pletz[2,3], M. Kitsera, F. Josa-Prado[4], S. Schild, C. Lembacher-Fadum[3], E. Dornisch, S. Roier, G. Gorkiewicz[5], I. Barasoain[4],J.I. Loizou[6], R. Breinbauer[3], J. Fernando Díaz[4], C. Högenauer[7]and E. L. Zechner*

[1]University of Graz, BioTechMed-Graz, Institute of Molecular Biosciences, Austria
[2] University of Graz, Institute of Chemistry, Austria
[3]Graz University of Technology, Institute of Organic Chemistry, Austria
[4]Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas (CIB-CSIC), Spain
[5]Medical University of Graz, Institute of Pathology, Austria
[6]CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Austria
[7]Department of Internal Medicine, Medical University of Graz, Austria
‡ These authors contributed equally
* For correspondence: ellen.zechner@uni-graz.at

The human gut microbiota constitutes a diverse community of trillions of microorganisms with a vast biosynthetic capacity.Microbiota-derived products have remarkably diverse chemistries with affinities for an equally diverse range of targets.They mediate both interactions with the host and with other microbes and performkey functions in promoting homeostasis and inthe onset and progression of diseases. Researchersfacean immense challenge, however, to identify causative relationships betweenmicrobial products and host phenotypes in this highly complex ecosystem.Despite that difficulty, our studies of enterotoxin-producing *Klebsiella oxytoca* have revealed a surprisingly simple model of disease-causing activity.
K. oxytoca is a typically benign resident of the human gut microbiota that also acts as a pathobiont. In some patients taking penicillin, expansion of this single organism results in antibiotic-associated hemorrhagic colitis (AAHC). We found that colitogenic strains carry a secondary metabolite biosynthetic gene cluster that is critical for the pathobiont to cause disease in an animal model of AAHC. To understand this pathology we determined the biosynthesis and structures of the cytotoxic products of the pathway. Tilivalline (TV) and tilimycin (TM) are non-ribosomal peptides belonging to the pyrrolobenzodiazepines. Wesynthesized the toxins chemically and then used high resolution HPLC-MS to demonstrate that both cytotoxins are produced in the human body.Quantitative analyses of mouse samples determined concentrations of toxinpresent in vivo during an active phase of AAHC. We then identified the different molecular targets of TM and TV and defined a detailed mode of action for each. Remarkably the data show that the *K. oxytoca* enterotoxin gene cluster produces distinct DNA damaging (TM) and microtubule stabilizing (TV) secondary metabolites. Although the cytotoxic functionalities of the enterotoxins differ, each substance triggered the apoptotic cell death characteristic for the colonic epithelium in AAHC. These findings illustrate the versatility of bacterial host interactions mediated by a single secondary metabolite biosynthesis pathway and provide novel insights into the molecular mechanisms of pathobiont activity.

CONSTRUCTION AND ANTIBACTERIAL EFFECT OF THE STRAIN ŽP – THE FIRST BACTERIAL CONJUGATION-BASED "KILL" - "ANTI-KILL" ANTIMICROBIAL SYSTEM

Starčič Erjavec M.[1], Petkovšek Ž.[1], Toplak N.[2], Kuznetsova M.V.[3], Maslennikova I.L.[3], Nekrasova I.V.[3], Žgur-Bertok D.[1]

[1] University of Ljubljana, Biotechnical Faculty, Department of Biology, Slovenia
[2] Institute of Ecology and Genetics of Microorganisms, *PFRC* Ural Branch Russian Academy of Science, Perm, Russia
[3]Omega d.o.o., Slovenia

Bacterial antimicrobial resistance is worldwide rising. Due to lack of efficient antimicrobial agents, resistance is responsible for deaths of thousands of patients infected with multi-drug resistant bacterial strains. Multi-drug resistant bacteria hence pose one of the greatest risks to human health. Thus, new antibacterial agents are urgently needed. One possible mechanism that can be used as an alternative to traditional antibiotic therapy is transfer of killing agents via conjugation. Our work was aimed at providing a proof of principle that conjugation-based antimicrobial systems are possible. We constructed a bacterial conjugation-based "kill"–"anti-kill" antimicrobial system employing the well known *Escherichia coli* probiotic strain Nissle 1917 genetically modified to harbor a conjugative plasmid carrying the "kill" gene (colicin ColE7 activity gene) and a chromosomally encoded "anti-kill" gene (ColE7 immunity gene). The constructed strain acts as a donor in conjugal transfer and its efficiency was tested in several types of conjugal assays: in liquid medium, in planktonic growth, in biofilm and in preformed biofilm (Starčič Erjavec et al., 2015). The efficiency of ColE7 bacterial conjugation-based antimicrobial system was further estimated by real-time PCR, flow cytometry and bioluminescence-based techniques (Maslennikova et al., 2018). On the basis of *traJ* gene expression in the killer donor cells, our results showed that the efficiency of the presented antimicrobial system against target *E. coli* was higher at 4 than at 24 h. Flow cytometry was used to indirectly estimate DNase activity of the antimicrobial system, as lysis of target *E. coli* cells in the conjugative mixture with the killer donor strain led to reduction in cell cytosol fluorescence. According to a *lux* assay, *E. coli* TG1 (pXen *lux*+ Ap^r) with constitutive luminescence were killed already after 2 h of treatment. Target sensor *E. coli* C600 with DNA damage SOS-inducible luminescence showed significantly lower SOS induction 6 and 24 h following treatment with the killer donor strain. As the constructed antimicrobial system showed a remarkable killing effect, it has the potential to be employed for prevention or treatment of *E. coli* infections.

THE LYTIC *MYOVIRIDAE* OF *ENTEROBACTERIACEAE* FORM TIGHT RECOMBINING ASSEMBLAGES SEPARATED BY DISCONTINUITIES IN GENOME AVERAGE NUCLEOTIDE IDENTITY AND LATERAL GENE FLOW

Tomaž Accetto[1] and Nika Janež[2]

[1]University of Ljubljana, Biotechnical faculty, Animal Science department, Slovenia
[2]Center of Excellence for Biosensors Instrumentation and Process Control, Slovenia

In *Bacteria*, a working consensus of species circumscription may have been reached and one of the most prominent criteria is high average nucleotide identity (ANI). ANI in effect groups strains that may recombine more or less frequently, depending on their biology, as opposed to rare interspecies gene transfer. For bacteriophages, which show various lifestyles, the nature of the fundamental natural unit, if it exists in a biological sense, is not well understood and defined. The approaches based on dot-plots are useful to group similar bacteriophages, yet are not quantitative and use arbitrarily set cut-offs. Here, we focus on lytic *Myoviridae* and test the ANI metric for group delineation. We show that ANI-based groups are in agreement with the International Committee on Taxonomy of Viruses (ICTV) classification and already established dot-plot groups, which are occasionally further refined owing to higher resolution of ANI analysis. Furthermore, these groups are separated among themselves by clear ANI discontinuities. Several approaches of recombination assessment (pairwise homoplasy index, clonal frame analysis, phylogenetic congruity) suggest that ANI delineated bacteriophage group members readily exchange core genes with each other while they do not with bacteriophages of other ANI groups, not even with the most similar. Thus, ANI-delineated groups may represent the natural units in lytic *Myoviridae* evolution with features that resemble those encountered in bacterial species (Accetto and Janež 2018).

Accetto T, Janež N (2018) The lytic *Myoviridae* of *Enterobacteriaceae* form tight recombining assemblages separated by discontinuities in genome average nucleotide identity and lateral gene flow. Microb Genomics 4: doi: 10.1099/mgen.0.000169

IDENTIFICATION AND CHARACTERIZATION OF PATHOGENS OF INFECTIOUS DISEASE WITH THE METHOD OF NEXT GENERATION SEQUENCING

Ivan Toplak[1], Darja Kušar[1], Bojan Papić[1], Jana Avberšek[1], Jože Grom[1], Peter Hostnik [1], Aleksandra Grilc Fajfar[1], Laura Šimenc[1], Mateja Pate[1], Danijela Rihtarič[1], Urška Zajc[1], Irena Zdovc[1], Tina Pirš[1], Petra Raspor Lainšček[2], Andrej Kirbiš[2], Urška Jamnikar Ciglenečki[2], Olga Zorman Rojs[2], Brigita Slavec[2], Uroš Krapež[2], Maja Rupnik[3], Sandra Janežič[3], Valerija Tkalec[3], Tanja Vrabič[3], Aleksander Mahnič[3], Minka Kovač[4], Anton Kovač[4], Nataša Toplak[4], Simon Koren[4], Matjaž Ocepek[1], Urška Kuhar[1]

[1]University of Ljubljana, Veterinary Faculty, Institute of Microbiology and Parasitology, Slovenia
[2]University of Ljubljana, Veterinary Faculty, Institute for Food Safety, Feed and Environment, Slovenia
[3]National Laboratory for Health, Environment and Food, Slovenia
[4]Omega consulting, engineering, development and research doo, Slovenia

Traditional microbiological methods, which are routinely used in clinical laboratories for identification and characterization of pathogens, have several important limitations as they are based on the cultivability of microbes. They are often time-consuming and restricted to a limited number of selected target species. Molecular methods brought significant improvements to the diagnostics of infectious diseases but they can only be used for determining the known pathogens. Next-generation sequencing (NGS) is based on parallel sequencing of large number of short sequences that are later assembled on the basis of overlapping reads. Within the project several strategies are using: the metagenomic approach, the identification of individual pathogens or their mixed populations in the samples. For diseases of viral etiology, the viral whole genome sequences of the economically important respiratory and enteric viruses have been determined to search for genetic characteristics related to the clinical manifestation of the disease. For bacteria, virulence and resistance determinants in addition to the possible role of animal or environmental isolates in human infections are investigated. The bacterial whole genome sequences methicillin-resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus pseudintermedius* (MRSP) are employed for phylogenetic analyses and studied as becoming an increasing problem in human and veterinary medicine. Since *Clostridium difficile* isolates previously associated with animal hosts are increasingly occurring in humans, these are also included. Project includes samples from animals with clinical manifestation of intestinal and respiratory diseases as being the most common cause of infectious diseases in humans and animals worldwide. Several whole genome sequences of viruses and bacteria have already been determined and new epidemiological connections between individual strains of pathogens have been investigated. The main results of project J4-8224 obtained during first year will be presented.


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CLOSING LECTURE

Borut Bohanec, University of Ljubljana, Biotechnical Faculty, Slovenia

Development of modern methods for breeding F1 hybrid cultivars of agricultural plants

DEVELOPMENT OF MODERN METHODS FOR BREEDING F1 HYBRID CULTIVARS OF AGRICULTURAL PLANTS

Borut Bohanec[1], Jernej Jakše[1], Katarina Rudolf[1], Nataša Štajner[1], Jana Murovec[1]

[1]University of Ljubljana, Biotechnical Faculty, Slovenia

Hybrid vigor has been demonstrated in the early 20th century after hybrid corn was invented. These discoveries lead to high yield increases in all major crops tested. Hybrids are preferred varietal forms since they can provide better yield, greater uniformity and faster identification of desired combinations of characters. Hybrid varieties are also preferred by breeding companies since the progeny of the next F2 generation from those F1 hybrids will segregate and therefore not consistently express the desired characteristics. Procedures to breed hybrid cultivar are more complex than breeding open-pollinated varieties or pure lines. Hybrid varieties are most often based on the crossing of two true breeding lines that should genetically complement each other. Often this complementarity of genotypes of genetically different parental lines in the F1 hybrid, results in a considerable improvement of e.g. growth characteristics, yield or adaptation to environmental stresses as compared to the individual parental lines and non-hybrid cultivars. Numerous methods have been proposed, the most common is based on development of large numbers of inbred lines originated form heterozygous parents by either self pollination or doubled haploid induction. Inbred lines are than tested for hybrid vigor often in two steps, by which first »general combining ability« and than »specific combining ability« is tested to identify a pair that expresses optimal characteristics. This standard procedure allows only a very limited number of line to line crosses since it is laborious and long lasting. Alternatives to this method are known, probably the most advanced among recently proposed was »reverse breeding« that requires genetic transformation to disable crossing over, doubled haploid induction, characterization of each chromosomal pair with adequate genetic markers and selection among lines to identify complementary lines. Method that utilizes random chromosomal pair distribution in gametes is limited to species with small chromosome number. Here we are proposing a new method that allows much higher number of line to line crosses as previously known. Our invention relates to innovative method of testing combining ability of inbred preferably doubled haploid lines, which is based on genotyping of each genetically diverse inbred line. In next step lines are intercrossed in to form F1 hybrid progeny. In the next season progeny is phenotyped on individual basis. For superior individuals parental lines are revealed by paternity testing, which due to homozygosity of lines and previous genotyping allows to identify both parental lines. By this method a much larger number of line to line testing can be achieved thus overcoming major bottleneck in breeding hybrid varieties. Details of the protocol and its utilization will be discussed.


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ABSTRACTS OF POSTER PRESENTATION


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GENETIC EVIDENCE OF INTRA-CULTIVAR VARIABILITY WITHIN ALBANIAN OLIVE CULTIVARS

Dervishi A.[1], Jakše J.[2], Ismaili H.[3], Javornik B.[2], Štajner N.[2]

[1]University of Tirana, Faculty of Natural Sciences, Department of Biotechnology, Albania
[2]University of Ljubljana, Biotechnical Faculty, Department of Agronomy, Slovenia
[3]University of Agriculture, Gene Bank, Genetic Resources Centre, Albania

Olive is one of the most important horticultural crops cultivated since antiquity in Albania. The generic denominations of olive cultivars based on morphological characteristics, such as the color, the size or the end-use of the fruit are the main causes of varietal disorder and the presence of synonymy and homonymy in many countries and worldwide olive collections. The identification of olive tree cultivars in Albania has been traditionally carried out by morphological and agronomical traits, which are influenced by environment. Sometimes the measured values are very similar increasing the risk of misidentification or mislabeling. To enable proper management of Albanian olive germplasm the identity analysis was performed in a set of 194 olive accession by means of 26 microsatellite loci. The SSR markers showed high information content (mean PIC=0.630) and low overall probability of identity (PI = 5.19×10^{-18}). 183 unique genotypes were identified, six groups of synonyms, nine groups of near synonyms and 50 cases of intra-cultivar variability and homonymy. A combination of only three loci enabled discrimination and identification of 83 % of genotypes analyzed. These three microsatellite markers may be very useful for rapid screening of Albanian or even other olive gemplasm in a collection.

The provided information on several cases of synonymy, intra-cultivar variability and homonymy not previously reported, indicating the high level of varietal disorder in Albanian olive germplasm is present, which should be re-evaluated and accurately resolved. On the other hand, this information is showing capability for selection of the best genotypes in these groups of plants.

DISEASE CAUSING MUTATIONS IN PATIENTS WITH BILARERAL NON-SYNDROMIC HEARING LOSS FROM NORTH-EASTERN SLOVENIA

Erjavec Škerget A.[1], Zagradišnik B.[1], Krgović D.[1,2], Golub A.[1], Rebolj J.[1], Kravos A.[1], Kokalj Vokač N.[1,2]

[1] University Medical Centre Maribor, Laboratory of Medical Genetics, Slovenia
[2]Faculty of Medicine Maribor, University of Maribor, Slovenia

Hereditary hearing loss is one of the most common sensory disorders worldwide. Mutations in the gap junction protein beta 2 (GJB2) gene are responsible for more cases of nonsyndromic recessive hearing loss (HL) than any other gene. The purpose of our study was to evaluate the characterisation and the prevalence of all, GJB2, GJB6 and other known mutations among affected individuals from North Eastern Slovenia. There were 33 individuals diagnosed with hereditary HL enrolled in the study. The allele specific PCR, MLPA technique by SALSA MLPA probemix P163, and the Sanger sequencing was used to screen the GJB2 coding region in all affected individuals. NGS sequencing with Oto-GeneSGKit® was used in 10 probands with bilateral, pre-lingual non syndromic HL with a negative medical history related to potential causes of acquired HL.
The overall diagnostic yield of the HL cohort was 8/33 (24.2%). The homozygous mutation c.35delG was identified in four out of 33 patients (12.1%). By five patients mono-allelic mutation c.35delG was detected (15.15%; 5/33). Three among them were compound heterozygotes: c.35delG variant compounded with variants: c.109G>A; WTIVS 1+1: G>A; and c.235delC. All pathogenic variants were confirmed by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method or Sanger sequencing.
NGS detected one homozygous disease-causing variant c.2464C>T in OTOF gene in one patient (10%), which is also heterozygote for the c.35delG.
The relative low ratio of individuals homozygous (12.1%) and heterozygous (15.15%) for the c.35delG mutation suggest that there are other genes responsible for nonsyndromic deafness in the North-Eastern Slovenian population. In one out of 10 tested probands with NGS this was confirmed.
Conclusion: c.35delG mutation in GJB2 gene is the most common HL causing mutation in North-Eastern Slovenia. The majority (87.5%) of the detected pathogenic genetic causes (7 out of 8 patients) were detected with MLPA technique which proved to be the most effective, reliable and economically acceptable technique for the detection of the genetic causes of GJB2 related deafness.

DETECTING RESISTANCE: USE OF dPCR FOR DETECTION AND QUANTIFICATION OF GANCICLOVIR RESISTANT HCMV

Alexandra Bogožalec Košir[1], Tašja Cvelbar[1,2], Mojca Milavec[1]

[1]National Institute of Biology, Department of Biotechnology and Systems Biology, Slovenia
[2]University of Ljubljana, Biotechnical Faculty, Department of Biology, Slovenia

Cytomegaloviruses are evolutionarily ancient viruses that belong to the herpesvirus family. Human cytomegalovirus (HCMV) or human herpesvirus 5 (HHV5) is a 230 kB double stranded DNA virus infecting monocytes, macrophages and dendritic cells. There are many diseases associated with HCMV such as hearing loss and abnormal development of immunocompromised foetuses, rejection and loss of graft, pancreatitis, hepatitis, cardiovascular disease in immunocompromised graft recipients, end-organ disease, colitis and retinitis in patients with AIDS and cardiovascular disease and proliferative disease (colon cancer, glioblastoma) in immunocompromised hosts. CMV often leads to wide spread of viral replication and dissemination to multiple organs and can be life threatening to individuals with compromised or immature immune system.
Antiviral drugs ganciclovir (GCV), cidofovir and foscarnet are used to treat patients and prevent further dissemination of the virus, of these the most widely used is GCV. The long-term treatment heightens antiviral pressure, which in turn heightens the occurrence of mutations. GCV targets UL54-gene encoding for viral DNA polymerase, however as GCV needs to be phosphorylated by phosphotransferase UL97, most resistant mutations occur in the UL97 region. These point mutations or single nucleotide polymorphisms (SNPs) represent 80% of all GCV resistance. As the development of resistance can be life-threatening especially for immunocompromised patients, a rapid method for detection and monitoring of infection and viral load is necessary.
The aim of our study was to select the most common SNPs that contribute to GCV resistance and design and characterise droplet digital polymerase chain reaction (ddPCR) assays that would enable faster and more accurate identification and quantification of resistant strains.

ATTITUDES AND AWARENESS OF POPULATION IN BOSNIA AND HERZEGOVINA TOWARDS GENOME EDITING, GENETIC TESTING AND GENETIC COUNSELING

Amina Agović[1], Azra Karić[1], Lejla Halilović[1], Sabina Semiz[1]

[1]International University of Sarajevo, Faculty of Engineering and Natural Sciences, Genetics and Bioengineering Program, Bosnia and Herzegovina

As the recent novel genomic discoveries are associated with more precise treatment options, the public engagement and education in this area seems to be particularly important. Here we explored the understanding and attitudes of Bosnian and Herzegovinian (BH) population towards these novel genomic technologies by using semi-structured interviews and a cross-sectional survey. Both questionnaires addressed three major topics, including genome editing, genetic testing and its importance in disease prevention, as well as the significance of genetic counseling in understanding results of genetic testing. In addition, we also explored the corresponding ethical issues related to these novel genomic tools. Our results show that the majority of the survey respondents (79%) believe that gene editing should be used in disease treatment, while 37% of them agree that the genome editing should be used for improving the human features. We also found that 41% of respondents are not aware of potential ethical issues associated with genome editing. Interestingly, more than half of the participants (58%) would undergo genetic testing and 72% of the survey participants believe that genetic counseling should be provided after these tests. In conclusion, our findings suggest that the novel genomic approaches in biomedicine deserve more active public engagement as well as additional educational programs in BH.

IDENTIFICATION OF A HOMOZYGOUS MISSENSE VARIANT IN THE *OTOF* GENE AT THE BOY WITH PRELINGUAL NON-SYNDROMIC SENSORINEURAL HEARING LOSS

A. Golub[1], A. Erjavec Škerget[1], B. Zagradišnik[1], J. Rebolj[3], D. Krgović[1,2], N. Kokalj Vokač[1,2]

[1]Laboratory of Medical Genetics, Universty Medical Centre Maribor, Slovenia
[2]Medical Faculty, Universty of Maribor, Slovenia
[3]University Medical Centre Maribor, Department of Otorhinolaryngology, Slovenia

Abstract

Identification of the etiology of inborn non-syndromic sensorineural hearing loss in children facilitates management and provides significant prognostic information. Until now a huge amount of progress has been made in understanding of the molecular basis of hearing and hearing loss. Despite the enormous heterogeneity of genetic hearing loss, variants in one locus, the *GJB2* gene, account for up to 50 - 60 % of cases of non-syndromic sensorineural hearing loss in some populations. *OTOF*-related deafness, designated as *DFNB9,* is characterized by two phenotypes: prelingual non-syndromic hearing loss and, less frequently, temperature-sensitive nonsyndromic auditory neuropathy. The diagnosis of *OTOF*-related deafness is confirmed by identification of biallelic deafness-related variants in *OTOF*, the gene encoding the protein otoferlin, on chromosome 2p23.3. In this report we will present the five-year-old boy with prelingual non-syndromic sensorineural hearing loss, at which we identified a homozygous missense variant in the *OTOF* gene. By next-generation sequencing (NGS) with Oto-GeneSGKit we discovered the homozygous missense variant c.2464C>T and an allele specific PCR was used for the conformation of this variant. The family segregation of the discovered variant by our patient will be also presented in this report. To date, only two more patients (two siblings) with this variant in the *OTOF* gene have been described in the literature.

Keywords: nonsyndromic autosomal recessive deafness - otoferlin - cochlear defect - case report

VARIABLE LEVELS OF TISSUE MOSAICISM CAN CONFOUND THE INTERPRETATION OF CHROMOSOMAL MICROARRAY RESULTS

Zagorac A[1], Golub A[1], Zagradišnik B[1], Erjavec Škerget A[1], Mujezinović F[2], Kavalar R[3] and Kokalj Vokač N[1,4]

[1]University Medical Centre Maribor, Laboratory of Medical Genetics, Slovenia
[2]University Medical Centre Maribor, Department of Perinatology, Slovenia
[3]University Medical Centre Maribor, Department of Pathology, Slovenia
[4]University Maribor, Medical Faculty Maribor, Slovenia

Introduction: Chromosomal microarray analysis has significantly increased the ability to diagnose medical conditions caused by copy-number variations. However mosaicism can confound the interpretation of chromosomal microarray results. We describe a case of sex chromosomes mosaic fetus with four cell lines. At the 22th week of pregnancy the hypoplastic left heart syndrome: presence of endocardial fibroelastosis of the left ventricle with aortic valve stenosis, was detected. Because of poor prognosis the pregnancy was terminated. Autopsy of the male fetus confirmed the presence of hypoplasia of the left ventricle and ascending aorta with VSD and mitral valve atresia.
Materials and Methods: QF-PCR test, aCGH analysis using 8x60K array and metaphase chromosome analysis after amniocenteses were performed.
Results: QF-PCR result was normal. Array CGH revealed additional copy of the whole short arm of chromosome Y in approximately 40% of cells and a loss of the whole long arm of chromosome Y: arr(Yp)x2[40],(Yq)x0. Metaphase analysis demonstrated mosaic karyotype: 45,X[82]/46,X,i(Y)(p10)[11]/47,X,i(Y)(p10),+i(Y)(p10)[7]/46,XY[2].
Conclusions: Our data demonstrate that although aCGH should be the first-tier test for clinical diagnosis of chromosome abnormalities, chromosome analysis remain valuable in the detection of mosaicism and delineation of chromosomal structural rearrangements. Phenotype of mosaic subject is primarily dependent on the dominant cell line in a specific tissue. Because our fetus was male we speculated that in gonads the cell lines i(Yp) and XY predominated. The high incidence of hypolastic left heart syndrome is seen in Turner syndrome girls, so the presence of 45,X cell line in our case could explain the heart malformation.
Array CGH is a useful method for identification of cytogenetic imbalances. For identification of various cell lines it is necessary to perform karyotyping.

EVALUATION OF CRISPR SYSTEMS FOR SINGLE NUCLEOTIDE CHANGE IN THE GENOME OF YEAST *Saccharomyces cerevisiae*

Blaž Lebar[1,2], Gašper Žun[1,2], Uroš Petrovič[1,3]

[1]Jožef Stefan Institute, Department of Molecular and Biomedical Sciences, Slovenia
[2]Faculty of Chemistry and Chemical Technology, Slovenia
[3]University of Ljubljana, Biotechnical Faculty, Department of Biology, Slovenia

Yeast *Saccharomyves cerevisiae* preferentially corrects double strand breaks through its powerful mechanism of homologous recombination. Nonetheless, non-homologous end joining (NHEJ) is a competitive process and resulting indels – insertions and deletions on both sides of the double strand brake – may present unwanted side products.
Key enzymes of CRISPR systems are programmable endonucleases, each orthologue with its unique features and mechanism of action. For instance, the so far most commonly used Cas9 of *Streptococcus pyogenes* creates blunt ends, while novel Cpf1 of *Francisella novicida* creates overhanging ends. Both their protospacer adjacent motif (PAM) sequence and structure of gRNA are distinctively different.
We applied abovementioned CRISPR systems on four divergent yeast *S. cerevisiae* strains. Additionally, we completely deleted the gene *NEJ1* in the reference yeast strain, as it is responsible for successful non-homologous end joining. Both endonuclease systems were designed against specific target in the coding region near SNP in the gene *IXR1*, responsible for oxidative stress tolerance, yet the SNP itself was not part of the crRNA sequence. As a linear matrix for homologous recombination a small region containing one of two possible SNPs was PCR-amplified.
Application of two distinctive diagnostic PCRs was required: (i) for testing the successful change in SNP and (ii) detecting potential indels in the area of cutting site, as PAM sequence was intentionally preserved. We detected the SNP site through specific annealing of only one of two primers with differences in length. Furthermore, we developed a diagnostic PCR for indels where four primers, two of them annealing one nucleotide over the cutting site, form two different products only if no NHEJ occurred.
After successful homologous recombination almost none of the indels were detected, independently of the yeast strain used. What is more, presence of *NEJ1* gene did not increase the NHEJ rate, resulting in general use of developed method. Conclusively, for the first time possibility to change only one specific nucleotide out of the whole yeast genome, using a simple and reproducible system, was shown.

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POPULATION STRUCTURE OF *Listeria monocytogenes* ISOLATES ASSOCIATED WITH ANIMAL RHOMBENCEPHALITIS IN SLOVENIA, 2008–2018

Bojan Papić[1], Nina Sluga[1], Mateja Pate[1], Darja Kušar[1]

[1]University of Ljubljana, Veterinary Faculty, Institute of Microbiology and Parasitology, Slovenia

Listeria monocytogenes is the causative agent of listeriosis, an important disease affecting both humans and animals with a high (>30%) mortality rate. Under EU regulations, all *L. monocytogenes* subtypes (clonal complexes, CCs) pose an equal health risk for humans and animals. However, several studies have shown that some CCs, e.g. CC1 and CC4, are strongly associated with a clinical origin and therefore represent a bigger threat.

We investigated 61 isolates collected from 2008 to 2018 from sheep, goat and cattle rhombencephalitis cases confirmed by histopathology. All isolates were characterized by PCR-serotyping and PFGE using *Asc*I and *Apa*I restriction endonucleases. Based on combined PFGE *AscI+Apa*I analysis, the isolates were assigned to their corresponding MLST CCs. Isolates belonging to CC1, CC4 and CC29 were further tested for invasiveness and replication in epithelial (Vero) and macrophage (BoMac) cell lines using the gentamicin protection assay. The isolates belonged to four serotypes (IVb, IIa, IIb, IIc) of which IVb (*n*=41) was the most prevalent. We identified 13 MLST CCs of which CC1 (*n*=28) and CC4 (*n*=7) were the most prevalent. CC1 prevailed in all animal species. In BoMac cell line, CC1 and CC4 were hyper-invasive in comparison to CC29. In both cell lines, CC4 was hyper-replicative in comparison to CC1 and CC29.

It has been shown before that CC1 is highly prevalent in animal rhombencephalitis and that its prevalence is significantly higher in rhombencephalitis than in the farm environment (Dreyer et al., 2016). Our results confirm CC1 as the most prevalent clone in animal rhombencephalitis, indicating its neurotropic nature. The gentamicin assay gave inconclusive results regarding the virulence potential of CC1, suggesting its health risk cannot be reliably assessed using *in vitro* gentamicin assay. A better understanding of the virulence potential of different clones could lead to implementation of more specific control measures in the context of food safety and animal/human health.

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A META-ANALYSIS OF THE PREVALENCE OF *Listeria monocytogenes* CLONES IN DIFFERENT CLINICAL FORMS OF LISTERIOSIS IN ANIMALS

Bojan Papić[1], Mateja Pate[1], Darja Kušar[1]

[1]University of Ljubljana, Veterinary Faculty, Institute of Microbiology and Parasitology, Slovenia

Listeria monocytogenes is an important zoonotic pathogen with a high mortality rate. The main three clinical forms of listeriosis in animals and humans are encephalitis/meningoencephalitis, abortion and septicaemia. In animals, the disease can also occur with a lower frequency as gastroenteritis, mastitis or ocular listeriosis. Several studies have reported CC1 and CC4 as the two most prevalent clones (MLST CCs) in both the diseased humans and animals. In CC4, a novel gene cluster LIPI-4 has been identified, which is involved in placental and neuronal tropism (Maury et al., 2016). However, the prevalence studies of *L. monocytogenes* clones taking into account the different clinical forms of listeriosis in animals are limited.

To identify the CCs which might be associated with a specific form of listeriosis, we have collected a large dataset (*n*=426) of animal clinical *L. monocytogenes* isolates with a known form of listeriosis originating from different animal species and different countries. The most prevalent clones were CC1 (32%), CC4 (12%) and CC37 (5%). In rhombencephalitis isolates, the prevalence of CC1 (48%) was significantly higher than in abortion isolates (16%). Prevalence of CC1-associated rhombencephalitis in small ruminants (47%) was not significantly different from the prevalence in cattle (53%) and was therefore independent of the animal species. In abortion cases, CC1 (16%) was also the most prevalent clone, but its prevalence was not significantly higher than the prevalence of CC37 (13%) or CC4 (12%). In other forms of listeriosis, the prevalence of CC1 was not significantly higher than the prevalence of other clones.

Our results confirm CC1 as the most prevalent clone in animal rhombencephalitis and suggest its neurotropism. CC1 and CC4 are known to be one of the most prevalent types in human listeriosis. However, in animal listeriosis cases, we observed a significantly lower prevalence of CC6 (4%), the second most prevalent clone in human listeriosis (Maury et al., 2016). Furthermore, a relatively high prevalence of CC37 in abortion cases in animals (13%) was surprising as it has been rarely reported in humans. To our knowledge, CC37 is the first reported clone specifically associated with abortion and should be further investigated to unravel the underlying mechanisms of its placental tropism.

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FUNCTIONAL *IN VITRO* CO-CULTURE MODEL ENABLES SIMULATION OF ANTI-TNF TREATMENT IN INFLAMMATORY BOWEL DISEASE PATIENTS

Boris Gole[1], Uroš Potočnik[1]

[1]University of Maribor, Medical faculty, Centre for human molecular genetics and pharmacogenomics, Slovenia

Inflammatory Bowel Disease (IBD) is a chronic autoimmune condition that may affect any part of the digestive system and seriously affects the quality of life. Biologicals, such as tumour necrosis factor inhibitors (TNFi) have vastly improved the treatment of IBD in recent years. Unfortunately, severe side effects, primary none-response and secondary loss of response are a serious issue in TNFi treatment. Accurate prediction of responsiveness to TNFi is therefore of utmost importance. So far however, no clinical marker satisfactorily predicts the response, while genetic background and gene expression data of non-responders are still under investigation.

With the "Omics" approach and subsequent gene ontology (GO) analysis of the putative biomarkers we and others are now identifying candidate molecular pathways contributing to the TNFi non-response.

To study in detail these candidate pathways we recently adopted an inducible *in vitro* model of immune and intestinal cells mimicking the *in vivo* interactions between the cell-types involved in the IBD. The model uses immortalized human cell lines such as Jurkat (T-cells capable of producing TNF, IFNγ and IL-2 upon stimulation) and HCT116 (colon carcinoma cells harbouring TNF receptor type 1) in a co-culture setting. This enables us to study the intracellular TNF signalling under controlled and repeatable conditions, as well as manipulation of the gene expression of putative TNFi non-response biomarkers.

Our preliminary results show that stimulation of cytokine production in immune system cells (Jurkat) indeed stimulates response (cell death) in the TNF-target colon cells (HCT116). Additionally, we show that C87, a small molecule inhibitor of TNF effective in murine cells is toxic for human cells.

CLONAL DIVERSITY AND SELECTED VIRULENT FACTORS OF EXTENDED-SPECTRUM AND AmpC-BETA-LACTAMASES-PRODUCING *Escherichia coli* ISOLATED FROM POULTRY FARMS IN SLOVENIA

Edita Šabić[1], Igor Gruntar[2], Olga Zorman Rojs[3], Jerneja Avguštin Ambrožič[4]

[1]University of Ljubljana, Biotechnical Faculty, Department of Food Science and Technology, Slovenia
[2]University of Ljubljana, Veterinary Faculty, Institute of Microbiology and Parasitology, Slovenia
[3]University of Ljubljana, Veterinary Faculty, Institute for Poultry, Birds, Small Mammals and Reptiles, Slovenia
[4]University of Ljubljana, Biotechnical Faculty, Department of Biology, Slovenia

The aim of our study was to investigate clonal diversity and selected virulence genes of extended-spectrum beta-lactamases (ESBL) and plasmid-mediated AmpC beta-lactamases (AmpC) producing *Escherichia coli* (*E. coli*) isolated during a longitudinal study from three broiler breeder flocks, four broiler flocks originating from these breeders, and four meat type turkey and their environment. Since the poultry houses were cleaned and disinfected before placement of birds all ESBL and/or AmpC producing *E. coli* isolates were introduced thereafter once or several times, probably from the environment. ERIC-PCR and the revisited "Clermont method" were employed to classify isolates into different clonal and phylogenetic groups, respectively. Resistance plasmids were isolated with a modified alkaline hydrolysis method. Additionally, all isolates were screened for the presence of virulence factor genes involved in adhesion, iron acquisition, toxin production, production of microcin V (*cvi*) and capsule synthesis. According to ERIC-PCR profiles, the studied 46 isolates could be assigned to 11 different clonal groups. In some flocks clonal diversity of isolates encoding bla_{SHV-12} was high at almost all sampling times, indicating a repeated bacterial entry into the flock and/or the persistence of a resistance plasmid, which was, in the ongoing study, recovered from three isolates, entering into different *E. coli* non-ESBL/AmpC strains. Interestingly, almost 45% of the isolates were from phylogenetic group A. The prevalence of virulence factor genes among the tested isolates was 91%, 13%, 18%, 29%, 25%, 15% and 1.5% for *fimH*, $ompT_{APEC}$, *kpsMT*II, *hra*, *fyuA*, *tsh*/*hbp* and *cvi*, respectively. Although several isolates were positive for hemolysis on BAB-agar supplemented with bovine blood, none of the known *E. coli* hemolysin genes could be confirmed by PCR, thus indicating the possibility of a new (virulence) gene.

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PROFILING OF QUANTITATIVE LATE BLIGHT RESISTANCE IN DIFFERENTIAL *R8* GENOTYPES

Eva Blatnik[1], Aleš Sedlar[1], Peter Dolničar[1], Vladimir Meglič[1]

[1]Agricultural Institute of Slovenia, Slovenia

Potato (*Solanum tuberosum* L.) is one of the most important food crops in the world and its production is highly threatened by a pathogen *Phytophthora infestans* which causes late blight. Conventional breeding for pathogen resistance relies on the use of genotypes with different sources of resistance. The genetic resistance to *P. infestans* in potato has been classified as race-specific or vertical (qualitative) and race-nonspecific or horizontal (quantitative) resistance. The vertical race-specific resistance is associated with dominant resistance (*R*) genes. Eleven dominant *R* genes were initially identified in the Mexican species *S. demissum* and introgressed by crossing and backcrossing methods into cultivated potato. However, deployment of these single genes did not provide durable resistance in the field due to the rapidly changing populations of *P. infestans* and appearance of new virulent strains of the pathogen overcame the resistance encoded by single race-specific *R* genes. One of the varieties with high quantitative resistance to late blight is Sarpo Mira, which contains five *R* genes: *R3a*, *R3b*, *R4*, *Rpi-Smira1* and *R8* gene. The goal of presented research was to obtain differential *R8* plants to study the quantitative resistance of Sarpo Mira caused by *R8* gene. 1230 crosses between Sarpo Mira and five late blight susceptible varieties were screened with molecular markers for the presence of *R3a*, *R3b*, *Rpi-Smira1* and *R8* genes. Only differential plants containing *R8* gene were selected and used for additional crossing with late blight susceptible varieties to obtain F1 population. To examine the mechanisms underlying quantitative resistance to *P. infestans*, the F1 population will be genotyped using molecular markers and phenotyped using infected detached leaves and whole infected plants. Resistance tests will be performed on plants grown in green-houses and experimental fields. Late blight resistance testing and selection of progeny will be performed by spraying the potato plants with a suspension of *P. infestans* genotypes isolated in Slovenia. Assessment of the late blight severity will be performed by visually estimating percent coverage of foliar late blight lesions. By using *P. infestans* effector assay we will be able to determine the presence of corresponding *R* genes in F1 population by observing a hypersensitive response caused by cell death (resistant phenotype) or a susceptible response. Combining these approaches will enable us to determine linkage between *R8* gene and quantitative resistance of Sarpo Mira.

CORRELATION OF GENETIC AND EPIGENETIC BIOMARKERS FOR PROGNOSIS OF TRIPLE NEGATIVE BREAST CANCER PROGRESSION AND INVASIVENESS

Helena Čelešnik[1], Dragana Bjelić[1], Monika Sobočan[2], Iztok Takač[2] and Uroš Potočnik[1]

[1]University of Maribor, Faculty of Medicine, Center for Human Molecular Genetics & Pharmacogenomics, Slovenia
[2]University Medical Centre Maribor, Division of Gynecology and Perinatology, Slovenia

BACKGROUND: Breast cancer (BC) is the second most common cancer in the world and the 5th leading cause of cancer-related death (1). The triple-negative breast cancer (TNBC) subtype, which is defined by the absence of estrogen (ER) and progesterone (PR) receptor expression and lack of amplification of epidermal growth factor-2 receptor (HER2) is associated with poor prognosis and lacks a well defined targeted therapy. This aggressive subtype is characterized by a high risk of relapse, metastasis and short progression-free as well as overall survival. Up to 50% of patients diagnosed with early-stage TNBC experience disease recurrence, and 37% die in the first 5 years after surgery (2). As determining the optimal treatment for TNBC patients is challenging, it would be beneficial to identify patients at high risk for disease progression at an early stage and adjust their therapy accordingly (3). The aim of our study was to evaluate the role of selected polymorphisms in immune signalling pathway genes as possible TNBC prognostic markers, and analyze correlations between these polymorphisms and changes at the epigenetic level.
PATIENTS AND METHODS: Samples from 100 breast cancer patients were obtained from the University Medical Centre in Maribor, Division of Gynecology and Perinatology (fresh blood and cancer tissue samples from BC/TNBC patients) and from the Department of Pathology (formalin-fixed paraffin-embedded tissue sections from TNBC patients). Healthy individuals (100 subjects) were analyzed as the control group. DNA isolated from patient and control samples was used for SNP genotyping by high resolution melting curve analysis or restriction fragment length polymorphism and for bisulfite conversion followed by methylation-specific HRM analyses for assessing DNA methylation.
RESULTS: Association analyses of selected single nucleotide polymorphisms in genes involved in immune signalling, including SNPs linked to the genes *CXCL12* and *GSDMA,* showed association with breast cancer progression (i.e. tumor grade, invasive ductal histology). The methylation status of these genes was analyzed for correlations with genetic polymorphisms.
CONCLUSIONS: The results of our study identified specific genetic biomarkers as useful predictors of disease progression in breast cancer patients.

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SMALL SECRETED PROTEIN VnaSSP4.2 STRONGLY INTERACTS WITH CARDIOLIPIN AND SULFATIDES

Helena Volk[1], Ingo Hein[2], Branka Javornik[1], Sabina Berne[1]

[1]Biotechnical Faculty, Department of Agronomy, Slovenia
[2]The James Hutton Institute, Cell and Molecular Sciences, United Kingdom

Verticillium nonalfalfae (*Vna*) is a soilborne plant pathogen, which causes devastating verticillium wilt disease on hop and other important crops. This study focuses on functional characterisation of a secreted effector protein, VnaSSP4.2, identified by Flajšman et al (2016) in the xylem sap of infected hop. *VnaSSP4.2* deletion mutants showed reduced virulence in susceptible hop, which was restored to the wild-type in the complementation lines. *Vna*SSP4.2 is a small 14.0 kDa protein without transmembrane domains and with a predicted prokaryotic membrane lipoprotein lipid attachment site.

Different approaches were combined in an effort to determine *Vna*SSP4.2's biological role. *VnaSSP4.2* was sub-cloned into pH7RWG2 and pH7WGR2 (N- and C-terminal mRFP fusion) (Karimi et al. 2007) and transiently expressed in *Nicotiana benthamiana* leaves after agroinfiltration. Confocal microscopy revealed nonspecific nucleo-cytosolic localization of both constructs. Yeast-two-hybrid (Y2H) screening with *VnaSSP4.2* as bait was performed against the potato cDNA library as prey, to identify possible plant protein targets. However, no protein interactors were found. The study subsequently focused on possible interactions between the recombinant protein *Vna*SSP4.2 and various lipids, as predicted by Prosite. The recombinant protein with N-terminal His_6 tag was expressed in *E. coli* BL21(DE3)pLysS and FPLC purified using a Ni-NTA column. Lipid binding tests on Membrane Lipid strips (Echelon) and Sphingo strips (Echelon) revealed that the recombinant protein *Vna*SSP4.2 strongly interacts with cardiolipin and sulfatides. Weaker interactions were also observed with phosphatidylglycerol, phosphatidylinositol (4)-phosphate, phosphatidylinositol (4,5)-bisphosphate and phosphatidylinositol (3,4,5)-trisphosphate. *Vna*SSP4.2 binding kinetics and affinity (Kd) for the selected lipids will be determined by surface plasmon resonance.

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IDENTIFICATION OF THE SPECIES OF ORIGIN OF MEAT PRODUCTS USING REAL TIME PCR: RESULTS OF FIVE YEAR TESTING IN SLOVENIA

Jelka Zabavnik Piano[1], Marko Cotman[1]

[1]University of Ljubljana, Veterinary Faculty, Institute for Preclinical Sciences, Slovenia

Various semi-quantitative methods for meat speciation were introduced in many EU official laboratories in February 2013 after the Food Safety Authority of Ireland announced that it had discovered horse DNA in a number of beef products in major supermarkets. This discovery caused coordinated testing on the authenticity of processed meat products across the EU countries – specially for the presence of horse meat. Species specific differences in the regions of mitochondrial DNA are ideal targets for speciation. In our laboratory we are using mitochondrial species specific DNA primers and real-time PCR to specifically amplify the fragments of horse, pig, cattle or poultry mitochondrial DNA to identify the meat species. Before 2013 meat species identification was not a common requirement in food processing industry or trade. However, since then food producers, supermarkets or official authorities are interested in knowing the species of animals from which the food is made. In 2013 we have performed 218 species identifications and from these 3 % of samples contained not declared meat. In 2014 the number of species identification testing increased to 266, in 4 % from these samples we detected not declared meat. Since then the number of samples is declining, reaching only 134 testing in 2017 (with 4 % of mislabelled meat species). In the majority of tested samples horse meat is the suspected not declared meat, however the most often mislabelled is pig meat, in 2015 it was detected in 12 from 26 tested samples (46 %) where the pig meat was not labelled on the declaration sheet. Food producers, supermarkets, official authorities and consumers are interested in knowing the species of animals from which the food is made in order to control and improve food production, to have control over suppliers, to prevent adulteration, because of specific health needs or for religious beliefs.

DIAGNOSTIC ALGORITHMS AND GENETIC TESTING FOR TWO BLOOD DISORDERS

Julija Lazarevič[1], Ana Marentič[2], Dejan Miskič[1], Nina Rupar[2], Špela Kert[3], Janja Murn[4], Maja Modic[1], Lucija Vermiglio[1], Nermin Jukan[5], David Zagoršek[5], Miha Moškon[5], Kristina Modic[6], Irena Preložnik Zupan[3,7] and Nataša Debeljak[8]

[1]University of Ljubljana, Faculty of Medicine, Slovenia
[2]University of Ljubljana, Biotechnical Faculty of, Slovenia
[3]University of Ljubljana, Faculty of Pharmacy, Slovenia
[4]University of Ljubljana, Faculty of Chemistry and Chemical Technology, Slovenia
[5]University of Ljubljana, Faculty of Computer and Information Science, Slovenia
[6]Slovenian Association of Patients with Lymphoma and Leukaemia L&L
[7]University Medical Center Ljubljana, Department of Hematology, Slovenia
[8]University of Ljubljana, Faculty of Medicine, Institute of Biochemistry, Slovenia

Objectives
The diagnosis of blood disorders with genetic background is challenging due to rapid development of new genetic tests and consequently updated diagnostic algorithms. We focused on two blood disorders, familial erythrocytosis (FE) and chronic myeloid leukemia (CML). CML is a myeloproliferative disease, diagnosed by detection of *BCR-ABL1* gene by FISH, response to treatment is monitored by RT-qPCR. FE is a rare inherited blood disorder defined by elevated numbers of red blood cells and hemoglobin. In contrast to CML diagnostic of FE is not well established, there is currently no diagnostic algorithm for diagnosis of FE in Slovenia.
The objective of the work was to create diagnostic algorithm for FE, to update in line with latest international guidelines the algorithm for CML and to develop a web based tool for their visualization. The development of the project proceeded through collaboration of students and tutors with different professional backgrounds: medicine, biotechnology, laboratory biomedicine, biochemistry and computer sciences in line with local environment (L&L).
Results
Overview of literature showed improved detection of the mutations causing resistance to treatment of CML. Genetic testing of FE is done by Sanger sequencing of most commonly involved genes and recently next generation sequencing (NGS).
We have taken into account the latest guidelines from the field of hematology in aim to propose new diagnostic algorithms for CML and FE. Algorithm for CML includes diagnosis, disease differentiation and therapy selection. For FE we extended the existing algorithm for polycythemia vera to include secondary and familial erythrocytosis, proposing several new diagnostic steps.
A web-based visualisation tool ViDis was developed to present the information. It enables creating, editing and viewing these diagrams. Visualisations can be constructed hierarchically, so the amount of information is small initially, but increases when zooming in within the selected segment.
Conclusion
We recognized need for development of additional genetic testing for FE and for some clinically important mutations in CML. Both algorithms are visualized within ViDis that is accessible to doctors, researchers and patients with FE and CML in Slovenia.

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DOES THE *VNA7.443* GENE ENCODE AN AVR EFFECTOR OF PHYTOPATHOGENIC FUNGUS *VERTICILLIUM NONALFALFAE*?

Klemen Dretnik[1], Kristina Marton[1], Helena Volk[1], Sebastjan Radišek[2], Branka Javornik[1], Sabina Berne[1]

[1]University of Ljubljana, Biotechnical Faculty, Department of Agronomy, Slovenia
[2]Slovenian Institute of Hop Research and Brewing, Department of Plant Protection, Slovenia

Effectors are generally defined as secreted molecules that modulate host cell structure and function to promote infection and/or trigger defence responses [1]. Avirulence (Avr) effectors are directly or indirectly recognized by cognate host resistance (R) proteins and trigger rapid and robust immune responses (ETI, effector triggered immunity), often associated with hypersensitive reaction [2]. Recent *Verticillium nonalfalfae* secretome profiling study [3] identified the candidate effector gene with high expression in roots of infected hop. *Vna7.443* knockout mutants did not exhibit any changes in fungal growth and sporulation, while pathogenicity in susceptible hop was similar to the wild type. In this work, we combined comparative genomics and subcellular localization studies with elicitor activity testing to decipher the molecular function of *Vna7.443*, which encodes putatively secreted 30.5 kDa neutral protein without cysteines. Predominantly unstructured protein with 16 aa signal peptide, has two internal repeats (at 21-122 aa and at 161-262 aa) predicted by SMART tool [4] and according to Conserved Domains search at NCBI belongs to the dnaA superfamily. Besides in *V. nonalfalfae* hop isolates, the *Vna7.443* gene was PCR-amplified in several other isolates of *Verticillium* including *V. alfalfae*, *V. dahliae*, *V. isaacii*, and *V. longisporum*. Blastp search against nr database at the NCBI revealed *Vna7.443* is a fungal-specific gene and its closest sequence homolog is BN 1708_009057 protein from *V. longisporum*. Significant expression of *Vna7.443* in roots of resistant ('Wye Target') and susceptible ('Celeia') hop during early stages of disease was determined by quantitative PCR analysis of infected hop samples, while six fold lower expression was observed in the stems. *Agrobacterium*-mediated transformation of *Vna7.443*-mRFP fusion construct, followed by imaging of *N. benthamiana* leaf sections under the fluorescence microscope, showed transiently expressed mRFP-tagged *Vna*7.443 protein in the plant nuclei. Since hypersensitive response was observed 5 dpi in agroinfiltrated leaves of *N. benthamiana*, elicitor activity testing of recombinant *Vna*7.443 in *Arabidopsis* and hop cell suspension cultures is underway. Yeast two-hybrid (Y2H) screening using *Vna7.443* as bait and cDNA library of infected hop as prey may reveal cognate R protein responsible for recognition of this Avr effector, while the corresponding R gene would advance hop resistance breeding to verticillium wilt.

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HEAD AND NECK CANCER SPHEROID CELL MODEL WITH STEM PROPERTIES

Larisa Zemljič[1],Boris Gole[1],Uroš Potočnik[1,2]

[1]University of Maribor, Faculty of Medicine, Center for Human Molecular Genetics and Pharmacogenomics, Slovenia
[2]University of Maribor, Faculty for Chemistry and Chemical Engineering, Laboratory for Biochemistry, Molecular Biology and Genomics, Slovenia

The preclinical cancer models predicting clinical treatment outcome are urgently required in early drug development, considering that mouse biology does not allow for an authentic model of human tumors to be generated. Since the complexity, heterogeneity, plasticity and diversity of the human tumor cannot be replicated in preclinical 2D tumor cell line models, 3D culture systems have been developed. They offer an unique opportunity to culture cancer cells in a spatially relevant manner, encouraging cell–cell interactions that cause the 3D-cultured cells to acquire morphological and cellular characteristics relevant to *in vivo* tumors. Previous studies demonstrated that cells cultures in 3D spheres may have "stemness" characteristics, since pluripotent cells tend to exhibit a sphere or colony morphology.
Because conventional therapies preferentially target cycling cells, quiescence of cancer stem cells (CSC) is thought to render them resistant to such treatment. Therefore the aim of our study is to design head and neck squamous cell carcinoma (HNSCC) tumor-spheres model with stem-like properties and design an assay for high throughput anti-CSC compound screening. For the tumor-sphere formation, single-cell suspensions are resuspended in culture media containing epithelial and basic fibroblast growth factors and plated in ultra-low attachment plates. After 7-10 days spheroids are formed. Cells from spheroids are labeled with fluorescence-conjugated antibodies against $CD44^{high}$ and $CD66^{low}$, a specific combination of biomarkers for HNSCC cells with stem properties.
Such model enables us to enrich cells with stem-like properties. Due to high reproducibility and low inter-experimental variability the designed model is suitable for high throughput applications, such as screening for novel bioactive compounds with anti-CSC activity.

microRNA EXPRESSION IN SKIN LESIONS OF ADULT PATIENTS WITH IgA VASCULITIS

Bolha L.[1], Hočevar A.[2], Tomšič M.[2,3], Jurčič V.[1], Pižem J.[1], Glavač D.[1]

[1]University of Ljubljana, Faculty of Medicine, Institute of Pathology, Slovenia
[2]University Medical Centre Ljubljana, Department of Rheumatology, Slovenia
[3]University of Ljubljana,Faculty of Medicine, Slovenia

Immunoglobulin A vasculitis (IgAV), formerly known as Henoch-Schönlein purpura, is a common form of childhood vasculitis, which also occurs in adults. IgAV is a small vessel vasculitis, clinically characterized by palpable purpura, arthralgia or arthritis, gastrointestinal and renal involvement. The disease is also accompanied by IgA deposits in the vascular wall, complement activation and large neutrophil infiltrates, which promote vascular inflammation. In patients with renal involvement, glomerulonephritis may develop, showing similarities to IgA nephropathy (IgAN), and can lead to end-stage renal disease. Overall, molecular basis of IgAV pathogenesis remains relatively unknown. There have been several reports associating dysregulated non-coding RNAs (ncRNAs), especially microRNAs (miRNAs), with dysfunction of the immune system and autoimmune diseases, including vasculitis. Nevertheless, little is known about miRNA expression in IgAV. Because skin manifestations are among the main hallmarks of the disease, we focused our study on miRNA expression in skin lesions from IgAV patient's shin area. The study included 65 skin samples from consecutive, untreated IgAV patients (61.5% male, median age 67.6 years, range 29–91) and 20 control normal skin samples from healthy individuals. For miRNA expression analysis, total RNA was isolated from skin tissue sections of formalin-fixed paraffin-embedded (FFPE) skin samples. By using qRT-PCR, we analyzed the expression of 10 miRNAs (miR-146a-5p, miR-148b-3p, miR-155-5p, miR-203-3p, miR-223-3p, miR-423-5p, let-7a, let-7b, let-7d and let-7f), several of which are skin-specific and/or were previously associated with vasculitis and inflammation. As demonstrated, the majority of analyzed miRNAs were significantly upregulated (2- to 19-fold (all $p<0.001$)), when compared to the controls. The exceptions were miR-148b-3p, which was significantly downregulated (2.2-fold, $p<0.001$), and miR-146a-5p, whose slight downregulation was not significant, when compared to healthy skin. In addition, we determined a significant correlation between the severity of purpura, granulocyte infiltration, skin necrosis, gastrointestinal tract involvement and several miRNA expression profiles in IgAV affected skin. Our results indicate that miRNA dysregulation plays an important role in IgAV-associated skin manifestations and that several analyzed aberrantly expressed miRNAs have the potential to be effectively used as novel biomarkers for IgAV.

PHARMACOGENOMIC TESTING CAN EXPLAIN THE OCCURRENCE OF ADVERSE EVENTS AFTER TREATMENT WITH SEVERAL DRUGS – A CASE REPORT

Maja Žugec[1], Tanja Blagus[1], Milojka Juteršek[2], Vita Dolžan[1]

[1]University of Ljubljana, Faculty of Medicine, Institute of Biochemistry, Farmacogenetics Laboratory, Slovenia
[2]Health Care Center Litija, Slovenia

Background
Pharmacogenomics explores associations between patient's genetic profile and patient response to drug treatment. The aim of preemptive pharmacogenomic testing is to achieve safer and more effective treatment.
Case presentation
The patient was 50 years old female with poorly controlled type 2 diabetes (T2D), well-controlled arterial hypertension, untreated hyperlipidemia and carotid atherosclerosis. In 2011 she reported severe gastrointestinal adverse events (AEs) after metformin treatment, therefore sulphonylurea was introduced. In 2012 the patient suffered from sulphonylurea induced hypoglycemia, therefore she was switched to insulin. In 2012 simvastatin was introduced, but she reported muscle pain, nausea, dyspepsia, flatulence and general weakness, leading to treatment discontinuaton. The patient opposed the introduction of new drugs because of severe AEs in the past. Due to high cardiovascular risk, the patient was advised again in 2017 to start with statin treatment. The patient agreed to participate in UPGx study) and to have her pharmacogenomic profile determined before treatment.
Methods
Patient's DNA sample was tested for pharmacogenomic UPGx panel using SNPline genotyping platform. In addition metformin transporters were genotyped.
Results
Patient had functional changes in six pharmacogenes that included genotypes: **CYP2D6*3/*4:** poor metaboliser of more than 20 drugs; **CYP2C9*1/*3:** decreased metabolism of suphonylurea, possible occurrence of hypoglycemia; **SLCO1B1:** decreased transport of simvastatin and atorvastatin, possible occurrence of AEs, fluvastatin recommended. The patient agreed to introduction of fluvastatin, but treatment was postponed due to newly diagnosed granulomatous lung disease. The extended PGx analysis showed heterosygosity for metformin transporters: **SLCA22A1 rs628031** GG>G**A**, and **SLCA47A1 rs2289669** GG>G**A**, both associated with higher risk for metformin AEs.
Conclusion
Pharmacogenomic testing can provide usefull information on patient's response to treatment with particular drug. If the pharmacogenomic testing in this patient's case was performed earlier, many severe AEs and ineffective treatments could be avoided.

PRIORITIZATION OF HIGH SENSITIVITY CALLS IN NEXT-GENERATION SEQUENCING VARIANT DISCOVERY

Mario Gorenjak[1], Katja Repnik[1,2], Gregor Jezernik[1], Staša Jurgec[1,2], Nataša Marčun Varda[3], Uroš Potočnik[1,2]

[1]Faculty of Medicine, University of Maribor, Centre for Human Molecular Genetics and Pharmacogenomics, Slovenia.
[2]Faculty of Chemistry and Chemical Engineering, University of Maribor, Laboratory of Biochemistry, Molecular biology and Genomics, Slovenia
[3]University Medical Centre Maribor, Division of Paediatrics, Slovenia

Rapid development of NGS technologies has revolutionized genetic research and allows to sequence the human genome in a matter of days. One sequencing run generates millions of fragmented DNA reads, which need further analysis. For variant discovery, appropriate pre-processing of data with prioritization of sensitivity over specificity is paramount in order to include as many potential variants as possible, without missing any. Here, we report a case of a low quality variant call, emitted due to employing prioritization of sensitivity over specificity, and found to be a causative variant for the patient's phenotype. DNA was extracted from the peripheral venous blood of a 5-year-old female with epileptic encephalopathy who presented with global mental retardation and a history of seizures. We generated sequencing libraries for paired-end exome sequencing on a MiSeq apparatus (Illumina) using TruSight One Sequencing Kit (Illumina). Generated reads were mapped to the UCSC hg19 reference genome using BWA and further analyzed using GATK and ANNOVAR program packages. The obtained call set was carefully inspected for variants in the *PCDH19* gene region, which is known to play a role in early infantile epileptic encephalopathy restricted to females. The inspected single nucleotide variants were either synonymous or flagged tolerated/benign according to the Sift and PolyPhen-2 algorithms. However, a heterozygous insertion was also called in the *PCDH19* target gene region, which was flagged as a low quality call and hence could be a possible false positive variant. The retained variant was "caught" in the final call set due to low calling emission threshold and higher sensitivity; it was also included in the annotation step. Annotation revealed that the called variant is a known insertion of the C base, with ID rs758946412 with the frameshift consequence p.Tyr366LeufsTer10, and is flagged in ClinVar as pathogenic with disease name "Early infantile epileptic encephalopathy type 9". Although the detected insertion is more than compliant with the patient's phenotype, validation is necessary in cases like this due to low read depth and high probability of false positive emission. Validation of the call was performed using SANGERseq and RFLP. Both analyses confirmed the heterozygous insertion rs758946412. The present case clearly elucidates the meaning of prioritization of sensitivity over specificity and supports the idea of lowering the emission thresholds.

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EVALUATION OF THE EFFICIENCY OF ColE7 CONJUGATION-BASED "KILL" - "ANTI-KILL" ANTIMICROBIAL SYSTEM FOR AVIAN PATHOGENIC *ESCHERICHIA COLI* (APEC)

Kuznetsova M.V.[1], Maslennikova I.L.[1], Gizatullina Ju.S.[1], Žgur-Bertok D.[2], Starčič Erjavec M.[2]

[1]Institute of Ecology and Genetics of Microorganisms, PFRC Ural Branch Russian Academy of Science, Russia
[2]University of Ljubljana, Biotechnical Faculty, Department of Biology, Slovenia

The aim of the study. As new antimicrobial agents are needed, in our study we evaluated the efficacy of the bacteriocin, colicin ColE7, bacterial conjugation based “kill” – “anti-kill” antimicrobial system for target recipient avian pathogenic *Escherichia coli* (APEC) strains, isolated from internal organs of infected broiler chickens.

Materials and methods. The ColE7 bacterial conjugation-based “kill” – “anti-kill” antimicrobial system is based on the genetically modified probiotic *E. coli* strain Nissle 1917, carrying the ColE7 activity gene (a DNase) on a conjugative F-plasmid derivative (pOX38a, Cm^r) and the ColE7 immunity gene (Gm^r) in the chromosome ("killer" donor strain). As a “control” donor strain, the *E. coli* strain N4i pOX38 – the Nissle 1917 strain with the ColE7 immunity gene integrated into the chromosome (Gm^r) and the conjugative F-plasmid derivative without the colE7 activity gene (pOX38, Cm^r) was used (Starčič Erjavec et al., 2015). Six APEC strains (Amp^r) isolated from the internal organs of infected broiler chickens were used as recipients. Conjugative transfer was carried out for 6 and 24 hours in plankton culture and in biofilm. The conjugation frequency was evaluated according to Guglielmetti et al. (2009).

Results. In experiments with a “control” donor strain, *E. coli* N4i pOX38, the frequency of conjugal transfer varied between 10^{-6}-10^{-2} and was higher in the developing biofilm than plankton at 6 h (1.73E–02 ± 2.24E–02 vs 2.27E–05 ± 2.40E–05 CFU/ml), and was comparable in both models at 24 h (4.45E–04 ± 5.46E–04 vs 6.25E–03 ± 8.63E–03 CFU/ml). In the latter case, the correlation (r) between the transfer of the plasmid in plankton and biofilm was 0.905. In conjugation experiments among the "killer" donor *E. coli* and the recipient APEC strains the transconjugants were not detected. All recipients that received the ColE7 gene via the conjugative transfer expressed it and were lysed due to colicin’s DNase activity.

The number of “control” and "killer" donors cells in the conjugation mixture after 24 hours of exposure was in plankton 2.22E+07 ± 2.03E+07 and 1.02E+08 ± 7.69E+07 CFU/ml, in the biofilm 7,38E+06 ± 2,33E+06 and 9,89E+06 ± 1,13E+07 CFU/ml, respectively, which indicates the high competitiveness of donor.

Conclusions. The *E. coli* strain possessing the ColE7 bacterial conjugation based “kill” – “anti-kill” antimicrobial system is promising as a basis for a probiotic preparation, which would be effective against APEC strains.

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GENOTOXIC ACTIVITY OF AFLATOXIN B1 EVALUATED IN 3D TISSUE-LIKE CELL MODEL

Martina Štampar[1], Jana Tomc[1], Metka Filipič[1] and Bojana Žegura[1]

[1]Department of Genetic Toxicology and Cancer Biology, National Institute of Biology, Slovenia

Liver cell lines cultured in two-dimensional (2D) monolayers are the most often used experimental system for investigating the adverse effects of xenobiotics *in vitro*. However, most hepatic cells cultivated in 2D lack relevant hepatic properties, mostly due to limited expression of drug metabolizing enzymes, which makes extrapolation of the results to *in vivo* conditions questionable. This is of particular importance in genotoxicity testing as unreliable results may lead to the requirement to be confirmed *in vivo* in animal studies. Lately, three-dimensional (3D) cultures, which better reflect *in vivo* conditions, are considered as an effective model for such studies. The aim of the study was to develop a 3D cell culture model with human hepatoma (HepG2) cell line and to validate the model using indirect-acting model genotoxic compound, aflatoxin B1 (AFB1). The 3D tissue-like cultures were prepared with forced floating method, cultured for three days and then exposed to non-cytotoxic concentrations of AFB1 (10, 20 and 40 µM) for 24h. After the exposure, the induction of DNA strand breaks was determined with the Comet assay. Expression of selected genes coding for phase I and II metabolic enzymes and DNA damage responsive was determined at mRNA level (qPCR) and confirmed at the protein level (Western blot). The results demonstrated dose dependent induction of DNA strand breaks at all applied AFB1 concentrations. DNA damage was associated with the upregulation of mRNA of DNA damage responsive genes *TP53, GADD45α* and *CDKN1A*. Moreover, AFB1 deregulated the expression of metabolic genes from phase I (*CYP1A2*, *CYP1A1* and *CYP3A4*) and phase II (*UGT1A1*, *SULT1A1*, *SULT1B1, NAT1* and *NAT2*), which was confirmed also at the protein level. These data confirmed the metabolic activity of the 3D *in vitro* cell model. Altogether, the results suggest that the newly developed 3D model with HepG2 cells has improved metabolic capacity compared to 2D model and has the potential for the application in genotoxicity studies as well as in regulatory testing of new chemicals and products.

GENE ELECTROTRANSFER MAY ELICIT PYROPTOSIS IN MURINE KERATINOCYTES

M Bosnjak[1], K Znidar[2], L C Heller[3,4] and M Cemazar[1,2]

[1]Institute of Oncology Ljubljana, Slovenia
[2]Faculty of Health Sciences, University of Primorska, Slovenia
[3]Frank Reidy Research Center for Bioelectrics, Old Dominion University, USA
[4]School of Medical Diagnostic & Translational Sciences, College of Health Sciences, Old Dominion University, USA

Intracellular pattern recognition receptors for the detection of molecular patterns typical of pathogens, including their genomes, are found in all mammalian cells. In several murine tumor [1, 2] and muscle cell types, increased expression and potential activation of DNA sensors, a subset of pattern recognition receptor, after empty plasmid DNA (pDNA) electrotransfer has been reported. Since skin is one of the important targeted tissues for electroporation based gene delivery, the aim of our current study was to evaluate the effects in murine skin cells.
An empty plasmid DNA (pDNA), gWiz Blank (Aldevron, Fargo, ND, USA), a plasmid serving as a backbone with a multiple cloning site for inserting a gene of interest for purposes of constructing therapeutic plasmids, was delivered into Kera-308 murine keratinocytes,. Electroporation was used as a delivery system for the introduction of this genetic material. Two different pulse protocols were compared, EP1: 600 V/cm, 5 ms, 6 pulses, 1 Hz and EP2: 1300 V/cm, 100 µs, 6 pulses, 4 Hz. Transfection efficiency and cytotoxicity were determined. The expression and upregulation of DNA sensors and downstream signaling molecules was determined. Caspase-1 activity was determined using The Caspase-Glo® 1 luminescence assay. Immunogenic cell death characteristic, such as HMGB1 and ATP release were studied. Changes in cell membrane such as phosphatidylserine (PS) exposure were also observed.
Among several DNA sensors expressed in Kera-308 cells, only DDX60, ZBP1/DAI, p202, p204 and AIM2 were upregulated after pDNA electrotransfer. Downstream signaling resulting in cytokine production was demonstrated. Activation of caspase-1 was also confirmed. As previously reported, EP1 pulses were more cytotoxic and caused extensive cell dead, which due to the caspase-1 activation may be pyroptosis. Other characteristics of pyroptosis, phosphatidylserine exposure on cell membranes and ATP release, were also determined.
The study showed for the first time that after gene electrotransfer cells may die by pyroptosis, which is a type of cell dead accompanied by the production of proinflammatory cytokines.

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BIOLOGICALY ACTIVE FOLATES ABOLISH THE HARMFUL EFFECTS OF METHYLENETETRAHYDROFOLATE REDUCTASE (MTHFR) POLYMORPHISMS IN HUMAN LYMPHOBLASTOID CELL LINES

Maša Vidmar[1,2], Alenka Šmid[2], Nataša Karas Kuželički[2], Irena Mlinarič-Raščan[2], Ksenija Geršak[1]

[1]University Medical Centre Ljubljana, Department of Obstetrics and Gynecology, Research Unit, Slovenia
[2]University of Ljubljana, Faculty of Pharmacy, The Chair of Clinical Biochemistry, Slovenia

Folate, also known as vitamin B9, is vital for cell development and growth, since it is involved in one-carbon transfer reactions essential for the synthesis of purines and pyrimidines. Its key role in human physiology is well known, as are various pathologies associated with low folate status (1,2). Therefore, it is not surprising that folic acid and its derivatives, e.g. 5-methyltetrahydrofolate (5-Me-THF) are among the most widely used food supplements. The aim of our study was to explore the consequences of folate deficiency and the importance of determining genetic polymorphisms, which influence the levels of biologically active folate, 5-methyl-THF (5-Me-THF). Human lymphoblastoid cell lines (LCLs) were chosen as a study model.

LCLs were cultivated in the classical RPMI 1640 media or in folate-deficient media for one week. Flow cytometry was used to analyze changes in cell cycle distribution. We found out that folate deficiency caused an arrest in the S phase of the cell cycle which was accompanied by the decrease in G0/G1 phase. Next, we investigated whether the addition of folic acid or 6-Me-THF to the folate-free media changes the distribution of cell cycle. We observed that S phase arrest is reversible when folate is added to the folate-deficient cells.

Folate deficiency in humans can occur due to the low dietary intake and also due to the genetic predisposition (1). The most investigated genetic polymorphisms are those in the *MTHFR* gene which influence the levels of biologically active folate. There are two common genetic polymorphisms that have been shown to decrease the activity of MTHFR, C677T (rs1801133) and A1298C (rs1801131) (3). In order to investigate whether these two polymorphisms modify the cell response to folic acid or 5-Me-THF, we used 35 LCLs with diverse genotypes of *MTHFR* gene. Cells with defective reduced folate carrier (RFC; SLC19A1; rs1051266 AA genotype) were excluded from the analysis. Cells were cultured in the presence of different concentrations of folic acid or 5-Me-THF and were evaluated for metabolic activity at 72 h. LCLs were divided in two groups according to expected enzyme activity: (1) LCLs with MTHFR activity higher than 60 percent (genotypes CC/AA, CC/AC, CT/AA, CC/CC), and (2) LCLs with enzyme activity lower or equal to 60 percent (genotypes TT/AA or CT/AC). The results showed that in cells with enzyme activity higher than 60 percent folate type did not influence the metabolic activity. However, this was not the case in the low MTHFR activity group. When treated with lower (≤ 25nM) concentrations of folates, higher metabolic activity was observed in LCLs treated with biologically active folate (5-Me-THF) vs. folic acid. This trend was lost when the cells were treated with higher concentration of folates.

Our findings indicate that genetic predisposition to folate deficiency can be bypassed by using biologically active forms of folate supplements.

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CYTOTOXIC AND GENOTOXIC POTENTIAL OF SELECTED TYROSINE KINASE INHIBITORS

Novak Matjaž[1], Kojc Kaja[1], Žegura Bojana[1], Filipič Metka[1]

[1]National Institute of Biology, Department of Genetic Toxicology and Cancer Biology, Slovenia

Potential risks associated with the release of pharmaceuticals into the environment are an important issue for the environmental regulators. Numerous studies confirmed contamination of the aquatic environment with various pharmaceuticals, among which anticancer drugs (ACDs) present especially high risk for indirectly exposed environmental organisms, mainly due to their mutagenic, genotoxic, cancerogenic and teratogenic characteristics. As the most important source of the release of ACDs into the environment was identified human excretion. Tyrosine kinase inhibitors (TKIs) are the group of ACDs that presents new era in chemotherapy with more targeted approaches and less side effects. There are currently around 20 drugs that target tyrosine kinases that have been approved for clinical use and hundreds more are undergoing clinical trials. These drugs are developed towards mutated tyrosine kinases of certain cancer types, which are responsible for tumor development and progression, including cell growth, differentiation, migration, and apoptosis. TKIs are designed to target specific kinases; however, they are not completely selective, and most of them have additional mechanisms of action at the cellular level, which may lead to undesired effects in non-target cells. The information related to potential genotoxic activity of TKIs is limited. Therefore, the aim of the present study was to evaluate genotoxic potential of three widely used TKIs, namely imatinib mesylate, regorafenib and dasatinib. As an experimental model we used zebrafish liver cells (ZFL), which are highly sensitive for detection of toxic and genotoxic effects of ACDs. The cytotoxicity was determined with the MTS assay and genotoxicity with the comet assay. The results showed that studied TKIs at non-cytotoxic concentrations induced DNA damage; however, at higher concentrations from those expected in the environment. Nevertheless, the study showed that in addition to the inhibition of tyrosine kinases, these inhibitors may interfere with DNA, presenting higher risk for the environmental organisms than expected. Therefore, there is an urgent need to assess their risk for the environment.

GENETIC CHARACTERIZATION OF EIGHT FULL-LENGTH ANTIBIOTIC RESISTANCE GENES *MYCOBACTERIUM TUBERCULOSIS* STRAINS WITH ION TORRENT NEXT-GENERATION SEGUENCING

Eva Sodja[1], Nataša Toplak[2], Simon Koren[2], Minka Kovač[2], Imogen Wright[3],4, Johann Eicher[3,4], Simon Travers[3,4], Manca Žolnir[1]

[1]University Clinic Golnik, Golnik, Slovenia,
[2]Omega d.o.o., Slovenia
[3]Hyrax Biosciences, Cape Town, South Africa
[4]South African National Bioinformatics Institute, University of the Western Cape, South Africa

Next-generation sequencing (NGS) can provide comprehensive analysis of *Mycobacterium tuberculosis* mutations that cause resistance to anti-tuberculosis drugs, which is a serious and growing threat to public health.
In our preliminary study we describe use of Ion Torrent technology for targeted full-length sequencing of selected *M. tuberculosis* genes.
We used NGS to analyze samples of ten clinical isolates from the National collection of clinical isolates of bacilli of tuberculosis and non-tuberculosis mycobacteria, University Clinic of Pulmonary and Allergic Diseases Golnik, Slovenia. Selected isolates were shown to be drug susceptible or mono-, poly- or multidrug resistant by phenotypic testing.
Eight genes related to antimicrobial resistance (*embB*, *eis*, *gyrA*, *inhA*, *katG*, *pncA*, *rpoB*, *rpsL*) were amplified in two highly multiplexed PCR reactions producing a total of 109 amplicons. NGS libraries were prepared automatically using the Ion Chef instrument and sequenced by the Ion S5 instrument. The sequencing results were analyzed manually, comparing the determined variants with published data and data available in the Tuberculosis Drug Resistance Database[1]. Furthermore, we tested a new online service Exatype™ for HIV and M. tuberculosis drug resistance analysis, which enables fast, accurate and user-friendly interpretation of NGS data for drug resistance genotyping. Finally, the resistance profiles obtained with both the manual approach and the Exatype™ platform were compared to the results of phenotypic drug susceptibility testing.
The next generation sequencing analysis was successfully used to predict drug-resistance profiles and there was high concordance between phenotypic and genetic results. Compared to the classical phenotypical approach, NGS offers two specific advantages. Firstly, depth of coverage that can be obtained allows for the detection of heteroresistance in mixed populations of resistant and susceptible organisms. And secondly, Ion Torrent sequencing approach shortens the time needed to obtain the resistance profiles from several weeks to just a few days.

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RADIOSENSITIZATION OF B16F10 MELANOMA AND TS/A CARCINOMA WITH GENE ELECTROTRANSFER OF PLASMID DNA ENCODING shRNA AGAINST MELANOMA CELL ADHESION MOLECULE

Simona Kranjc[1], Maša Bošnjak[1], Valter Mrak[2], Gregor Serša[1,3], Maja Čemažar[1,4]

[1]Department of Experimental Oncology, Institute of Oncology Ljubljana, Slovenia
[2]Angelini Pharma d.o.o., Slovenia
[3]Faculty of Health Sciences, University of Ljubljana, Slovenia
[4]Faculty of Health Sciences, University of Primorska, Slovenia

Radiotherapy is principal treatment of tumors and metastases. To improve the efficacy, irradiation can be combined with adjuvant treatments, such as chemotherapy, vascular targeted or immunomodulatory. Vascular targeted gene therapy already proved to have radiosensitizing effect in preclinical studies using shRNA molecules against endoglin (Savarin et al: Cancer Gene Ther, 23:214-20, 2016 and Radiol Oncol, 51:30-9, 2017). To extent these studies, the aim of the current study was to determine, whether gene electrotransfer (GET) of plasmid encoding shRNA against melanoma cell adhesion molecule (MCAM; pMCAM), can radiosensitize murine B16F10 melanoma and TS/A carcinoma.

In vitro, radiosensitivity of melanoma and carcinoma cells after GET of pMCAM was determined using clonogenic assay. *In vivo* B16F10 melanoma, growing in C57Bl/6 mice and TS/A carcinoma, growing in BALB/c mice, were treated by triple GET and single dose irradiation giving 1 day after the first GET. The antitumor effect was determined by tumor growth delay assay and tumor cures. In addition, histological analysis in tumors (proliferation, apoptosis, necrosis, vascularization, hypoxia, infiltration of immune cells) were used to evaluate the underlying mechanisms of antitumor activity.

Melanoma was significantly more responsive to radiosensitization with gene silencing of MCAM than carcinoma. This effect cannot be ascribed to the intrinsic sensitivity of cells since *in vitro* the effect was reversed, higher enhancement factor of combined treatment was obtained in carcinoma cells. Silencing of MCAM combined with irradiation affected tumor vasculature, induced hypoxia, apoptosis, necrosis and reduced tumor cell proliferation. In addition, the significant increase of infiltrating immune cells was observed in all groups combined with irradiation in melanoma, while in carcinoma only after combined treatment, indicating the important role of immune response. Immune cell infiltration after combined treatment (also using control instead of therapeutic plasmid) was significantly higher in melanoma than in carcinoma, which correlated with the antitumor response, being higher in melanoma than in carcinoma.

In conclusion, gene electrotransfer of plasmid silencing MCAM radiosensitize melanoma and carcinoma, predominantly due to direct effect on tumor vasculature and tumor cells. However, the release of tumor associated antigens after irradiation activated immune response, resulting in increased antitumor effectiveness, which was more pronounced in melanoma than in carcinoma.

HAPLOID INDUCTION OF CANNABIS (*Cannabis sativa* L.) THROUGH POLLINATION WITH IRRADIATED POLLEN – PROGRESS REPORT

Svetik Sinja[1] , Flajšman Marko[1], Bohanec Borut[1]

[1]University of Ljubljana, Biotehnical Faculty, Department of Agriculture, Slovenia

Homozygous inbred lines of cannabis is difficult to obtain due to inbreeding depression and flowering characteristics. The aim of our work was to develop a protocol of haploid induction from female gametes of *Cannabis sativa* trough pollination with irradiated pollen. Technique would be useful in breeding F1 hybrid varieties and in genetic research.

Embryo development is stimulated by pollen germination on the stigma and growth of the pollen tube within the style but irradiated pollen is unable to fertilize the egg cell. It has been used successfully in several species.

Two cultivars ('Finola' and 'Tiborszallasi') were included in pollen germination

were isolated from these treatments, but only one regenerant was formed. For this reason lower experiments. Several media compositions were tested of which two were most suitable. Optimal irradiation treatment of pollen was as follows: extracted pollen mixture was treated in a petri dish with increased air humidity.

Results revealed that high X-ray irradiation doses such as 619 Gy and 530 Gy were to damaging to pollen. Even though the pollen germinated *in vitro* and embryos were formed. 232 embryos doses - 442 Gy and 354 Gy were applied. Isolated embryos from 354 Gy treatment developed without any signs of damage, 90 embryos were induced and 25 of them formed regenerants. Pollinations with 442 Gy treatments fromed 164 embryos of which 56 developed into regenerants. Preliminary results of ploidy test with flow cytometry showed the diploid status of all regenerants, except for two which were tetraploids. Homozygosity testing by SSR markers is in progress to reveal somatic or gametic origin of regenerants.

TISSUE CULTURE OF MEDICAL CANNABIS (*Cannabis sativa* L.)

Špela Mestinšek Mubi[1], Marko Flajšman[1], Borut Bohanec[1], Jana Murovec[1]

[1]University of Ljubljana, Biotechnical Faculty, Department of Agronomy, Slovenia

ABSTRACT

Micropropagation is a technique of rapid *in vitro* clonal propagation of plants, which enables their high-frequency multiplication. The success of micropropagation depends on several factors, such as the composition of the culture medium, growth environment and genotype. Since there are only a few reports about tissue culture of *Cannabis sativa* L. (Feeney and Punja, 2003; Lata et al., 2009; Chaohua et al., 2016; Lata et al., 2016), the development of an efficient micropropagation procedure may be of high commercial value for the medical cannabis industry. The study was performed as part of MGC Pharmaceuticals project "Breeding medical cannabis (*Cannabis sativa* L.)" in collaboration with the Biotechnical Faculty of the University of Ljubljana. Four experiments were done on their breeding lines MX-CBD-11 and MX-CBD-707 as follows:

1.) In the first experiment, sterilized nodes were inoculated on three different culture media. The number and the length of newly developed axillary shoots were evaluated 30 days after inoculation. The results showed that in the breeding line MX-CBD-11 the basal MS medium supplemented with meta-Topoline induced the higher number of axillary shoots and the longest shoots, while in the breeding line MX-CBD-707 the higher number of axillary shoots were induced on MS basal medium without hormones. The longest shoots were formed on the MS basal medium supplemented with meta-Topoline.

2.) In the second experiment, we studied the effect of node position on initiation of shoot cultures. Nodes from five positions were inoculated on MS basal medium without hormones. As in the previous experiment, the number of developed axillary shoots and their lengths were evaluated 30 days after inoculation. For both breeding lines, nodes closer to the apical meristem produced less axillary shoots than those from distal ones.

3.) The results of the third experiment showed that the average number of shoots per node is increasing through the time.

4.) The last experiment aimed to evaluate the effect of 10 different culture media, composed of basal MS medium supplemented with BAP and IAA at various concentrations, on *in vitro* culture initiation. In this experiment, the breeding line MX-CBD-11 produced a higher number and longer axillary shoots than the breeding line MX-CBD-707 on all tested media.

Our study showed that *in vitro* culture of medical cannabis can be initiated on different culture media and that nodal position and genotype have a significant influence on the success of shoot culture establishment. **Keywords:** *Cannabis sativa* L., medical cannabis, micropropagation

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CYP51A1 SNPs AND THEIR EFFECT ON HUMAN HEALTH

Tadeja Režen[1], Iza Ogris[2], Marko Sever[2], Franci Merzel[2], Simona Golic Grdadolnik[2], Damjana Rozman[1*]

[1]University of Ljubljana, Faculty of Medicine, Centre for Functional Genomics and Bio-chips, Institute of Biochemistry, Slovenia
[2]National Institute of Chemistry, Ljubljana, Slovenia

CYP51A1 is an enzyme involved in cholesterol biosynthesis which is an essential pathway involved in development, growth and maintenance. *Cyp51a1-/-* knockout is embryonically lethal, while heterozygous *Cyp51a1+/-* mice are healthy and fertile. Therefore, we could expect to find heterozygous individuals also in human population. Aim of this work was to evaluate *CYP51A1* variants in view of their effect on enzymatic activity and on human health.
With the onset of whole genome sequencing, new human *CYP51A1* single nucleotide polymorphisms were reported. Search in SNP databases revealed less than 200 polymorphisms with missense change of amino acid and majority of them were designated as tolerated or benign by SIFT and PolyPhen-2. Polymorphisms designated as potentially damaging are very rare polymorphisms and in some cases reported only in one family. Only few polymorphisms were so far associated with a phenotype in humans. These are congenital cataract, neonatal hepatic failure, life span, hypertension, C-HDL level and spontaneous premature labour. From potentially damaging polymorphisms, we selected three missense polymorphisms to evaluate their effect on enzymatic activity *in vitro* and *in silico*. Proteins with R277L and R431H polymorphism were expressed *in vitro* but we failed to measure a P450 spectrum. Next, we performed molecular dynamic simulations to test how amino acid change affects CYP51A1 interactions with its substrate lanosterol and obligatory redox partner POR in comparison to wild type protein. Simulations predicted that variant R431H and D152G would have lower binding potential towards lanosterol with larger distance between lanosterol and haem and different formation of hydrogen bonds with lanosterol. Calculations of binding enthalpy revealed that variant D152G has almost zero binding enthalpy towards redox partner followed by variant R277L.
In conclusion, we propose to include selected damaging *Cyp51a1* variants into diagnostics panels of selected diseases.

MOLECULAR MECHANISMS OF FLUOROPYRIMIDINE TOXICITY BEYOND *DPYD* – CASE REPORT

Tanja Blagus[1], Nežka Hribernik[2], Nataša Šarvari Snoj[2], Marko Boc[2], Vita Dolžan[1]

[1]University of Ljubljana, Faculty of Medicine, Institute of Biochemistry, Pharmacogenetics Laboratory, Slovenia
[2]Institute of Oncology Ljubljana, Slovenia

Background Fluoropyrimidines (FP) are antimetabolite used in the cancer treatment, especially for treating colorectal and breast cancer. Although 5-fluorouracil (FU) and its prodrug capecitabine (CA) are widely prescribed, they can lead to serious, potentially life threatening adverse events (AEs). Individuals who carry genetic polymorphisms in dihydropyrimidine dehydrogenase (*DPYD*) are at high risk for severe toxicity, however toxicity may also occur in patient with normal DPD activity. The aim of this study was to investigate if genetic polymorphisms affecting FP metabolism beyond *DPYD* can explain FP toxicity.
Case presentation Three patients were referred for pharmacogenomic testing due to development of serious FP-induced toxicity. **Patient 1 (P1)** was a 35 years old female with breast cancer treated with FU. She was hospitalized due to grade 4 (G4) febrile neutropenia, G3 thrombocytopenia, G2 anaemia and G1 stomatitis. **P2** was a 52 years old male with colon cancer who received CA in adjuvant treatment. He was re-hospitalized after the first treatment cycle with CA due to G2/3 gastro-enterocolitis, G2/3 hand and foot syndrome (HFS) and sepsis. **P3** was a 68 years old female diagnosed with metastatic breast cancer who experienced severe CA-toxicity: G4 febrile neutropenia, G3/4 mucositis-stomatitis, G2 HFS and sepsis.
Methods All patients were tested for four most common functional *DPYD* polymorphisms. In addition, five SNPs in three genes involved in FP pathway previously reported to be associated with FU and/or CA toxicity were genotyped: ***MTHFR*** (methylenetetrahydrofolate reductase), ***TYMS*** (thymidylate synthase) and ***CDA*** (cytidine deaminase).
Results and discussion DPD deficiency was confirmed only in **P1** who was a compound heterozygous (71679G **C**A; A2846T **T**A), resulting in decreased DPD activity and reduced FU clearance. She also had polymorphisms in *MTHFR* A1298C; **C**A, *CDA* -31delC: C/**del** and *TYMS:* **3R**/**3R**. **P2** and **P3** had normal *DPYD* genotype, however they carried several other polymorphisms; **P2**: *MTHFR C677T* **TT**, *CDA -31delC* **del**/**del**, *CDA K27Q* **C**A, *TYMS* 2R/**3R** and **P3**: *CDA -31delC* **del**/**del**, *CDA K27Q* **CC**. According to previous reports, both SNPs in *CDA* may explain severity of CA induced AEs, as they may lead to *CDA* overexpression in the process of CA activation and likely higher amounts of circulating FU.
Conclusion Our results indicate the importance of extended testing for polymorphisms in genes involved in FP pathway for explaining FP-induced AEs.

CYTOSOLIC DNA SENSORS AND CYTOKINES ARE INDUCED IN DOSE- AND TIME- DEPENDENT MANNER AFTER IRRADIATION OF B16F10 MOUSE MELANOMA CELLS

Tanja Jesenko[1], Katarina Žnidar[2], Ajda Prevc[1], Maša Bošnjak[1], Katja Uršič[1], Gregor Serša[1,5], Loree C. Heller[3,4], Maja Čemažar[1,2]

[1]Institute of Oncology Ljubljana, Department of Experimental Oncology, Slovenia
[2]University of Primorska, Faculty of Health Sciences, Slovenia
[3]Old Dominion University, Frank Reidy Research Center for Bioelectrics, USA
[4]Old Dominion University, School of Medical Diagnostic & Translational Sciences, College of Health Sciences, USA
[5]University of Ljubljana, Faculty of Health Sciences, Slovenia

Local irradiation (IR) is a standard cancer treatment. Aside from direct cytotoxic effect via lethal DNA damage, IR also impacts the phenotype of tumor cells, alters the tumor microenvironment and consequently enhances activation of the immune system, which could lead to bystander and abscopal effects of IR. Activation of cytosolic pattern recognition receptors, i.e. DNA sensors and subsequent induction of cytokines could be one of the mechanisms causing these effects. The main producers of cytokines after IR were reported to be immune cells, however they can also be produced by tumor cells. Therefore, the aim of this study was to investigate whether IR causes upregulation of cytosolic DNA sensors in B16F10 melanoma cells and whether this leads to cytokine release.

B16F10 mouse melanoma cells were irradiated with graded doses (0.5, 2, 4, 6, 8 Gy) and cell survival was determined by clonogenic assay. The presence of DNA in the cytosol after IR was determined by immunofluorescence labelling. RNA was purified 5, 24, 48 or 72 hours after IR of cells and thereafter q-PCR was performed to determine the expression of some cytosolic DNA sensors. The expression of IFNβ1 and TNF-α was measured at the mRNA level by q-PCR and at the protein level by immunofluorescence labelling.

The survival of melanoma cells was already reduced at IR with 0.5 Gy and only 4% of the cells survived at IR with 8 Gy. The presence of cytosolic DNA after IR was confirmed. IR of the tumor cells increased the expression of cytosolic DNA sensors DDX60, DAI/ZBP1 and p204 in a time- and dose-dependent manner. Namely, the expression of all three cytosolic DNA sensors was significantly elevated in B16F10 melanoma cells at 24 h after IR and expression reached peak at 48 h after IR with? doses higher than 4 Gy. At 72 h post-IR, the expression of cytosolic DNA sensors decreased to the levels of control-unirradiated cells. The expression of cytokines, IFNβ1 and TNF-α was upregulated on mRNA and protein levels at doses higher than 4 Gy.

The results of our study demonstrate that IR can trigger the production of IFNβ1 and TNF-α via the sensing of DNA released into the cytosol after irradiation in B16F10 melanoma cells, which could be involved in the bystander and abscopal effects of radiation.

COMPARATIVE PROTEOMIC ANALYSIS OF DROUGHT-STRESS RESPONSIVE PROTEINS IN COMMON BEAN (*Phaseolus vulgaris* L.) CHLOROPLASTS

Tanja Zadražnik[1], Jelka Šuštar-Vozlič[2]

[1]Biotechnical Faculty, Agronomy Department, Slovenia
[2]Agricultural Institute of Slovenia, Crop Science Department, Slovenia

Drought is one of the major stress factors that limit agricultural productivity of many crops, including legumes and among them common bean (*Phaseolus vulgaris* L). The mechanisms of drought stress response and tolerance in common bean at molecular level is still not well characterized. The response to stress is a complex mechanism due to physiological and molecular changes of plants. One of the major organelles, whose functions are affected during drought stress are chloroplasts. Here, we report the proteomic approach with two-dimensional differential in gel electrophoresis (2D-DIGE) for analysis of drought-responsive proteins in chloroplast leaves of two cultivars differing in their response to drought, Tiber and more sensitive Starozagorski čern. Plants for proteome analysis were harvested on days 6 and 13 after the beginning of water withdrawal. Chloroplasts from control and drought-stressed plants leaves were isolated using Percoll gradient centrifugation. The yield of isolated chloroplasts in drought-stressed samples of both cultivars was lower compared to control samples in both cultivars. The intactness of the isolated chloroplasts was checked and used for protein isolation. To estimate protein contaminants in the chloroplast proteins, western blot analysis was performed. Proteins from four biological replicates for each treatment conditions were labelled with G-Dyes developed for fluorescence 2D-DIGE technology. The DeCyder bioinformatic software was used for quantitative and statistical analysis of the 2D-DIGE images, which is currently ongoing. Differences in protein abundance between control and stressed plants and between the cultivars will be compared. The results of the research will provide the basic insight into the molecular regulatory mechanism of chloroplast proteins in common bean under drought.

QUANTIFICATION OF GENETICALLY MODIFIED SOYBEAN IN COMPLEX SAMPLES USING DIGITAL PCR

Alexandra Bogožalec Košir [1,2], Tina Demšar [1], Dejan Štebih [1], Jana Žel [1], Mojca Milavec [1]

[1]Department of Biotechnology and Systems Biology, National Institute of Biology, Slovenia
[2]Jožef Stefan International Postgraduate School, Slovenia

Real-time quantitative polymerase chain reaction (qPCR) has been the golden standard for detection and quantification of GMOs for almost two decades. The quantification of GMOs with qPCR method is based on the reference material standard calibration curve, which is sensitive to inhibitors especially in complex samples. Digital PCR (dPCR) is an advanced method, where the reaction mixture is divided into many individual reactions called partitions, enabling absolute quantification without the need for a reference material standard curve and is less sensitive to partial inhibition.
The objective of this study was to investigate the potential of dPCR to overcome the difficulties of GMO quantification by qPCR in complex samples. As previous studies have shown that direct transferability of qPCR-validated methods to a dPCR system is possible, we have transferred two simplex dPCR assay targeting the soybean species-specific gene and one of the most abundant soybean lines on the world market, commercially known as Roundup Ready® soybean. Additionally, we have merged these two simplex assays in one duplex dPCR assay to enable even more time and cost efficient analysis. The analysis were performed on BioRad's ddPCR platforms QX100™ and QX200™. The assays were assessed on certified reference materials and complex real-life feed samples. To investigate the influence of extraction, two common DNA extraction techniques were performed.

THE DIVERSE AND EXTENSIVE PLANT POLYSACCHARIDE DEGRADATIVE APPARATUSES OF THE RUMEN AND HINDGUT *PREVOTELLA* SPECIES: A FACTOR IN THEIR UBIQUITY?

Tomaž Accetto[1] and Gorazd Avguštin[1]

[1]University of Ljubljana, Biotechnical faculty, Animal Science department, Slovenia

The *Prevotella* are commonly observed all along the mammalian gut starting already at the oral cavity. Their abundance rises dramatically in parts of the gut dedicated to plant biomass degradation such as the rumen and the hindgut both in culture as well as in the NGS based studies. One of the three human enterotypes is characterized by high *Prevotella* abundance and they account to up to 10 % in chimpanzee and even more in pig hindgut and in the rumen studies. Despite that, the knowledge on their actual role, though postulated to lie in soluble fibre degradation is scarce and the *Prevotella* diversity in these habitats, though obvious, is currently not appreciated. Additionally, the *Prevotella* are diverse taking the roles from pathogens in oral cavity to mutualists in the rumen. Thus, when reporting simply the share of (16S rRNA gene) NGS reads affiliated to genus *Prevotella,* one may report bacteria of quite heterogeneous and in the case of hindgut strains currently unknown ecological roles. Here we use a collection of many novel *Prevotella* species obtained by us and through Hungate 1000 project to expand insight into the rumen/hindgut derived strains. We show by genomic analysis that 23 rumen/hindgut *Prevotella* species (of which 16 are novel) generally possess extensive repertoires of polysaccharide utilization loci (PULs) and carbohydrate active enzymes targeting various plant polysaccharides. These PUL repertoires separate analysed *Prevotella* into generalists and specialists yet a finer diversity among generalists is evident too, both in range of substrates targeted and in PUL combinations targeting the same broad substrate classes. The growth experiments largely corroborated the above bioinformatic predictions supporting the nutrient niche partitioning as a major factor in cohabitation of *Prevotella* species. Upon evaluation of the shares of species analysed in this study in rumen metagenomes we found firstly, that they contributed significantly to total *Prevotella* abundance and secondly, we observed unexpected conservation of their shares in metagenomes originating from sites as apart as New Zealand and Scotland. In addition, *Prevotella* species, which were isolated from human and pig hindgut, as well as certain oral human *Prevotella*, were found to consistently contribute to these (ruminal) metagenomes indicating frequent passage between different hosts.

PHD MRNA EXPRESSION IN NON-SMALL CELL LUNG CANCER PRIMARY TUMOR TISSUE IS DECREASED AND IT CORRELATES WITH DISEASE STAGE

Tomaž Krumpestar[1], Matija Rijavec[1], Tanja Čufe[1]r, Peter Korošec[1], Ana Koren[1]

[1]University Clinic of Respiratory and Allergic Diseases Golnik, Slovenia

ABSTRACT

Prolyl hydroxylase domain (PHD) proteins play critical role in maintaining oxygen homeostasis with their major function being the detection of intracellular oxygen. PHD family consists of three members: PHD1, PHD2 and PHD3. Different PHD isoforms have different contribution to specific pathophysiological processes including tumorigenesis and cell growth. The aim of this study was to determine *PHD* mRNA expression in tumor and matching normal lung tissue of operable non-small cell lung cancer (NSCLC) patients and to correlate it with clinical characteristics of patients including disease stage.

Primary lung tumor tissue was obtained from 61 patients with operable NSCLC undergoing radical surgery. In 23 out of 61 patients, matching morphologically normal lung tissue was also obtained. Tissue was collected immediately after surgery and stored in RNA*later* RNA stabilization reagent. After RNA extraction, *PHD1*, *PHD2* and *PHD3* mRNA expression in tissue samples was quantified by RT-qPCR.

The median age of patients was 62 years (range 42-79 years) and 34 of 61 (56%) were male. The majority of patients were current or former smokers (50/61; 82%). Of the 61 patients, 35 (57%) had adenocarcinoma, 21 (34%) had squamous cell carcinoma histology and 5 (8%) were classified as NOS (Not Otherwise Specified). All patients were diagnosed with operable disease (stage I-III) and were treated with radical surgery. *PHD1* mRNA expression in NSCLC tumors was significantly decreased compared to matching normal lungs (P<0.0001). Similarly was observed for *PHD2* expression (P<0.0007) but not for *PHD3* expression (P<0.786). Significant correlations between *PHD1* (stage I vs. III P=0.0067; stage II vs. III P=0.018) and *PHD2* (stage I vs. III P=0.0418) expression levels and disease stage were also found in our study. Contrary, no significant correlation between disease stage and *PHD3* expression was found.

In this study we found that *PHD1* and *PHD2* mRNA expression levels in primary NSCLC tumors are decreased. Furthermore, *PHD1* and *PHD2* expression levels were also found to correlate with disease stage, showing the importance of PHD proteins in lung cancer development. Further studies are needed to determine the possible prognostic and predictive role of PHD proteins in NSCLC.

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IDENTIFICATION OF microRNAs AND THEIR TARGETS IN HOP PLANTS (*Humulus Lupulus* L.) IN RESPONSE TO *Verticillium nonalfalfae* INFECTION

Urban Kunej[1], Jernej Jakše[1], Maja Mikulič Petkovšek[1], Nataša Štajner[1]

[1]University in Ljubljana, Biotechnical Faculty, Department of Agronomy

Micro-RNAs (miRNAs) are involved in gene silencing processes at the post-transcriptional level, thereby regulating a wide variety of biological processes and also ensure a fine-tuned regulation of genes involved in defence mechanisms during stress response and defence against pathogens. Limited knowledge is available on the miRNA population of hop (*Humulus lupulus* L.) which is highly susceptible to verticillium wilt, a disease that threatens European hop production. The unique pathosystem of hop plant and fungus *Verticillium nonalfalfae* is an attractive model to study phenolic and miRNA response during infection.

In our study, we conducted artificial inoculation of two hop varieties Celeia (susceptible to verticillium wilting) and Wye Target (tolerant) with lethal strain T2 of *V. nonalfalfae*. A sampling of 18 repetitions (3 control and 15 treated plants) per variety was performed at 1, 3, 6, 12, 15, 18 and 30 days post-inoculation (DPI). The colonization pattern of *V. nonalfalfae* during infection was obtained by quantitative PCR (qPCR) using *V. nonalfalfae* specific primers. Furthermore, we used HPLC-MS method to quantify phenolic compounds responsive to colonization with *V. nonalfalfae*. Fungal colonization and phenolic compound profiles help us to select the most appropriate sampling points for miRNA-Seq experiment and to characterize and identify the responsive differentially expressed miRNAs during verticillium wilt pathogenesis.

Fungal colonization and phenolic compound profiles show increasing colonization in stems of susceptible variety CE, while in the resistant variety it is not noticeable. On average, a higher total phenolic content was found in infected and control samples of susceptible variety CE compared to resistant variety WT. In a preliminary miRNA-Seq analysis of infected and control WT root samples from 1 DPI, we identified 981 miRNA candidates in draft hop genome. Of the identified, 144 miRNA candidates mapped to 46 known miRNA gene families from miRBase. Using DESeq2 we identified 24 differentially expressed miRNA families of these 3 are known (miR-169, miR-160, miR-399) and 21 are novel.

Identified responsive hop miRNAs will be validated using RT-qPCR. We will perform identification and computational functional analysis of hop genes/transcripts, which are targeted by selected identified hop miRNAs. Finally, genome-wide analysis of hop targets, which are cut by the action of miRNA silencing, will be performed.

CIRCADIAN CLOCK AROUND THE LIPIDS AND MICROBIOTA: FINDINGS FROM CELL LINES <NO LIMITS>

Urša Kovač[1], Andraž Mulej[2], Matej Kociper[2], Jasmina Livk[2], Katka Pohar[2], Katja Malovrh[1], Marjanca Starčič Erjavec[3], Damjana Rozman[1], Alojz Ihan[2]

[1]University of Ljubljana, Faculty of Medicine, Institute of Biochemistry, Center for Functional genomics and Bio-Chips, Slovenia
[2]University of Ljubljana, Faculty of Medicine, Institute of Microbiology and Immunology, Slovenia
[3]University of Ljubljana, Biotechnical faculty, Department of Biology, Slovenia

Circadian rhythm, the most widely investigated biological rhythm, modulates nearly every mammalian physiological process, also the immune system. It is broadly accepted that microbiota plays a fundamental role in shaping the host immune system, which has an extraordinary capacity to adapt and respond to highly diverse challenges. The bacterium *Escherichia coli* is a well-known member of the microbiota and plays a key role among the facultative anaerobe bacteria in the microbiota.

The aim of the study was to assess the role of selected *Escherichia coli* (*E. coli*) strains from healthy human gut microbiota that have extraintestinal pathogenic *E. coli* (ExPEC) virulence factors in influencing the circadian rhythm. Our goal was to show that *E. coli* strains, possessing ExPEC virulence associated genes, from the gut microbiota of healthy people are capable of triggering the host (cell line) circadian clock and lipid metabolism trough complement pathway activation.

Different cell lines (Huh7, HepG2, Caco-2, U2OS) were routinely grown at 37 °C and 5% CO_2 in a humidified atmosphere in DMEM containing 10% of FBS and 1% of penicillin/streptomycin. *E. coli* strains were grown under aerobic conditions in LB medium in an incubator-shaker at 37 °C. *Overnight E. coli* cultures were transferred into fresh LB medium (1:100 dilution), grown till the early stationary phase and used for infection of different cell lines. 24 hours prior to inoculation with *E. coli*, complete medium used for the cell lines was changed to medium lacking antibiotics and serum. Cell lines in medium lacking antibiotics and serum were subsequently infected with selected *E. coli* strains. Total RNA samples were collected every 2h for consecutive 24 h and subjected to qRT-PCR analysis for circadian clock genes expression. To evaluate the effect of selected *E. coli* strains we performed a cosinor analysis.

It was shown that *E. coli* from gut microbiota of healthy humans that possess virulence associated genes are capable of modulating the host's immune system, in particular complement system even to the intensity of activation of pathogenic strains. Some *E. coli* strains that bind complement factor H were able to avoid immune system, also lipopolysaccharide profile played an important role in the complement response. Since immune system and circadian rhythm are greatly interconnected our next goal is to define weather the *E. coli* strains that are capable of binding C7 and/or factor H differently affect circadian rhythm and weather the number of virulent factors plays a potential role in maintaining circadian rhythm in our co-culture as well.

COMPERATIVE EVALUATION OF *PHASEOLUS COCCINEUS* L. GERMPLASM ORIGINATING FROM EIGHT CENTRAL EUROPEAN COLLECTIONS

Barbara Pipan[1], Jelka Šuštar-Vozlič[1], Vida Todorović[2], Marina Antić[2], Brezeanu Creola[3], Sonja Ivanovska[4], Mirjana Vasić[5], Wolfgang Kainz[6], Fabiano Miceli[7], Pavol Hauptvogel[8], Roberto Papa[9] Vladimir Meglič[1]

[1]Agricultural Institute of Slovenia, Crop Science Department, Slovenia
[2]University of Banja Luka, Genetic Resources Institute, Bosnia and Herzegovina
[3]University of Agricultural Science and Veterinary Medicine, Vegetable Research and Development Station Bacau, Romania
[4]Ss. Cyril and Methodius University of Skopje, Faculty of Agricultural Sciences and Food, Macedonia
[5]Institute of Field and Vegetable Crops, Vegetable Crops Department, Serbia
[6] Austrian Agency for Health and Food Safety, Seed Collection, Austria
[7]Universita degli studi di Udine, Dipartimento di scienze agroalimentari, ambientali e animali, Italy
[8]National Agricultural and Food Science, Research Institute of Plant Production, Slovakia
[9]Facoltà di Agraria, Dipartimento di Scienze degli Alimenti, Italy

In the last decade several thousand accessions were collected in different parts of Europe and are stored in national gene banks; at present the Phaseolus Database as part of the EURISCO Web catalogue contains over 46000 records. Main objective of the study (ECPGR_SMARTLEG project) was proper phenotypic and genetic evaluation of European Phaseolus accessions with emphasis to *Phaseolus coccineus* germplasm providing new data to EURISCO and AEGIS. During the project, we provide three different types of datasets including morho-agronomic traits of plants, morphometric seed characteristics and genetic profiles of *P. coccineus* accessions from eight Central European geographic origins; Slovenia, Romania, Bosnia and Herzegovina, Serbia, Italy, Slovakia, Macedonia and Austria. Before sowing, the morphometric characterization of *P. coccineus* seeds was performed according to adopted Community Plant Variety Office-Technical Protocol and Phaselieu/AIS descriptors observing 14 different seed characteristics. The morphological evaluations were performed upon adjusted descriptors for Phaseolus (ECPGR_PhasChar from Austria) under field conditions in Slovenia, Romania, Serbia, Macedonia and Bosnia and Herzegovina. A set of 12 already proven and reliably cross-species amplified nSSR markers among *P. vulgaris* and *P.* coccineus genomes was applied to evaluate genetic structure of *P. coccineus* genotypes. Morpho-agronomical evaluation shows differentiation between *P. coccineus* accessions even for standard varieties under geographically distinct field conditions. Global principal component analysis extracted four components which cumulatively explains 74, 6 % of morphometric variability of *P. coccineus* seeds. Analysis of molecular variability on the basis of allelic patterns reflects 3 % of the molecular variability among *P. coccineus* germplasm from eight different geographic origins which means that 97 % of germplasm is common to all accessions ($p>0.01$). Regarding to genetic structure of the *P. coccineus* germplasm from eight geographically distinct collections, three genetic clusters were formatted; the average genetic distance between genotypes in each cluster varies between 0.592 and 0.816. Overall results indicate to a common geographic origin of *P. coccineus* accessions from different collections within Central Europe.

CONJUGATIVE ANTIBIOTIC RESISTANCE PLASMIDS IN *Escherichia* coli ISOLATED FROM POULTRY

Žana Lovšin[1], Olga Zorman Rojs[2], Irena Zdovc[3], Jerneja Ambrožič Avguštin[1]

[1]University of Ljubljana, Biotechnical faculty, Department of Biology, Slovenia
[2]University of Ljubljana, Veterinary faculty, Institute for Poultry, Birds, Small Mammals and Reptiles, Slovenia
[3]University of Ljubljana, Veterinary faculty, Institute of Microbiology and Parasitology, Slovenia

The aim of our study was to determine the presence of conjugative plasmids (CP) encoding antibiotic resistance genes and the distribution of these plasmids among 85 *Escherichia coli* isolated from dead or culled birds submitted to pathological examination and their environment.
First the isolates were assigned to phylogenetic groups as described by Clermont in 2000 by PCR. The conjugation experiments were carried out on BHI-agar plates, using $J53^{AziR}$ as the recipient strain. Transconjugants (TKs) were selected on six different LB-agar plates supplemented with sodium azide and one of the following antimicrobials: ampicillin (Amp), tetracycline (Tet), chloramfenicol (Cam), trimethoprim (Tmp), ciprofloxacin (Cip) or cefotaxime (CTX). TKs isolated on one selective antimicrobial were additionally cross-tested for the resistance against other used antimicrobials to determine whether all resistance determinants were present on the same conjugative plasmid.
Out of 85 isolates, 48,2 % were carrying one of the eleven, according to different combinations of resistance genes, identified types of conjugative plasmids. Most prevalent were plasmids encoding only Ap^R genes. Among ESBL/AmpC producing *E.coli* in 34,2 % of the isolates 4 different types of plasmids were confirmed. Among non ESBL/AmpC isolates plasmids could be confirmed in 56,8 % environmental isolates and 39 % isolates from dead or culled birds. Regarding phylogenetic groups, most isolates with CP belong to group B1 (39 %), followed by A_1 (24,4 %), D_2 (19,5 %), A_0 (14,6 %) and D_1 (2,4 %). None of the five $B2_3$ isolate had a CP. Interestingly only group A_1 isolates had CPs carrying Tmp and Cip resistance genes. Screening for non-antimicrobial plasmid-mediated phenotypic markers revealed three D_2 group isolates, all carrying conjugative plasmid encoding three of four resistances, that were hemolytic on BAB-medium supplemented with bovine blood. None of the known *E. coli* hemolysins could be confirmed by PCR. Our study revealed a high proportion of *E. coli* strains carrying conjugative plasmids among poultry and their environment, representing a high risk for additional mobilization and spreading of critical resistance genes (e.g. for ESBL/AmpC, karbapenemases) and virulence factor genes via horizontal gene transfer.

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UNVEILING RNAI IN PHYTOPATHOGENIC FUNGI *VERTICILLIUM NONALFALFAE*

Taja Jeseničnik[1], Nataša Štajner[1], Jernej Jakše[1]

[1] Biotechnical Faculty, Department of Agronomy, Slovenia

RNA interference is a major gene silencing mechanism modulating gene expression at a post-transcription level in many eukaryotic organisms. However, the role of RNAi core components and small RNAs remains poorly understood in phyto-pathogenic fungi. *Verticillium nonalfalfae* is a soil born plant pathogen causing verticillium wilt in many important crops worldwide. Two pathotypes, mild strain and lethal strain, have been isolated from Slovenian hop fields, with the lethal strain causing severe symptoms in hop plants, resulting in intense withering and complete dieback of the plant. Here, with the accessible genome sequence and detailed transcriptome of the two strains, we report on identification and characterization of the three core components of RNAi (Argonaute, Dicer and RNA-dependent RNA polymerase), followed by RT-qPCR expression analysis and phylogenetic analysis. Protein sequences for core RNAi genes available for different fungal species in UniProtKB database were used as reference sequences for BLASTX against annotated *V. nonalfalfae* genome. Hits with highest e-values were selected for further analysis. RNA sequencing data were mapped on selected sequences for manual gene model curation and protein sequence prediction was performed using NCBI ORFfinder. Potential RNAi genes were further characterized for their functional domains using Conserved Domain Database, Pfam and SMART tool. Total of two Argonaute, two Dicer and two RNA-dependent RNA polymerase genes were identified and RT-qPCR was performed to determine their expression levels in four different fungal tissues: spores, mycelia, mycelia grown on simulated xylem fluid medium and resting mycelia. Phylogenetic analysis was performed among fungal plant pathogenic species representing Ascomycota taxonomic group to determine relationships based on RNAi core components. Moreover, fungal specific small RNAs were isolated and sequenced from both fungal pathotypes and microRNAs prediction is in progress.

PROGNOSTIC SIGNIFICANCE OF TRANSCRIPTION FACTOR NANOG IN SQUAMOUS CELL CARCINOMA OF THE ORAL CAVITY

Gašper Grubelnik[1], Emanuela Boštjančič[1], Nina Zidar[1]

[1] Institute of Pathology, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia

NANOG is an important transcription factor involved in a complex regulation of cell death and proliferation and has a major role in determining cell fate in human development and cancer. It is believed to be silenced after birth, it remains silenced or low-expressed in adult tissues [1, 3]. However, it has been shown in a variety of cancers that expression of NANOG can be detected in a proportion of cancer cells that exhibit stem cell-like properties (cancer stems cells, CSCs) [1]. It has been postulated that CSCs can trigger malignant transformation, progression and development of metastases. As a result, expression of NANOG has been reported in different cancer types, including squamous cell carcinoma (SCC) of the oral cavity [1, 2]. The aim of our study is to analyze the expression of NANOG and its protein regulators, OCT4 and SOX2, in oral SCC with lymph node metastases (N+) in comparison to those without lymph node metastases (N0) to determine whether they can be used as prognostic markers.

We have obtained samples of 24 patients with oral SCC that have been divided in two groups: 13 patients with oral SCC N0 and 11 patients with oral SCC N+. For all 24 patients, tumor samples and tumor-adjacent normal tissue have been available. RNA and DNA have been extracted from tissue samples. Expression of NANOG and its postulated protein regulators, SOX2 and OCT4, has been analyzed using quantitative real-time polymerase chain reaction. Methylation status of promoter region of NANOG has been analyzed using Sanger sequencing.

Analysis has not shown upregulation of NANOG and OCT4 in N0 group of oral SCC, whereas SOX2 has shown slight up-regulation. However, up-regulation of all three genes, NANOG, OCT4 and SOX2 has been found in N+ group of oral SCC. Moreover, both, NANOG and OCT4, but not SOX2, have been found to be up-regulated in group N+ compared to group N0 of oral SCC. We did not find any differences in methylation status in promotor region of NANOG in tumor samples compared to tumor-adjacent normal samples, neither in N0 nor in N+ group of oral SCC. Our results suggest that mRNA expression of NANOG and OCT4 could be correlated to each other and that NANOG expression is not dependent on methylation status in oral SCC. Differential expression of NANOG and OCT4 in oral SCC with lymph node metastases compared to those without lymph node metastases indicates that NANOG and OCT4 could be used as prognostic markers.

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DCN, *EPHA4*, *FN1*, *SPARC*, *SPON2* AND *SPP1* GENES ARE POSSIBLE BIOMARKERS DISTINGUISHING COLORECTAL ADENOMAS FROM CARCINOMAS

Margareta Žlajpah[1], Emanuela Boštjančič[1], Nina Zidar[1], Nina Hauptman[1]

[1] Institute of Pathology, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia

Colorectal cancer (CRC) arises from epithelial cells as a result of genetic and epigenetic changes. Since CRC is one of the most common type of cancer worldwide, bowel cancer screening programs have been introduced in several different countries [3]. Since introduction, a large number of early lesions is identified including adenomas with epithelial misplacement (benign) as well as malignant adenomas (early carcinomas). Despite well-defined morphological features of adenomas with epithelial misplacement and early carcinomas [2], there is a significant number of problematic cases of (pre)cancerous lesions with ambiguous features leading to different diagnostic opinions among pathologists [1, 4, 5]. An appropriate treatment is based on accurate diagnosis, therefore specific biomarkers for distinguishing between benign and malign lesions of CRC could be used as an additional conformation.

Using public functional genomics data repository (gene expression omnibus, GEO), we have found several projects with gene expression profiles of normal, adenoma and carcinoma colonic samples acquired from microarray experiments. Applying *in silico* methods we have identified sixteen differentially expressed genes between adenomas and carcinomas. All sixteen genes have been clustered in groups based on their cell function with DAVID platform (database for annotation, visualization and integrated discovery). One group represents the proteins of extracellular matrix (DCN, SPP1, SPON2, SPARC, EPHA4 and FN1) that have been recognized as possible biomarkers. We have analyzed the expression of these genes using quantitative real-time polymerase chain reaction. We have found down-regulation or same expression as in normal mucosa in adenomas and up-regulation in carcinomas for all six genes that have been experimentally validated. Our experimental results confirm that all six genes are differentially expressed between adenoma and carcinoma. To test these genes as possible biomarkers for distinguishing adenoma with epithelial misplacement from early carcinoma further experiments should be done on a larger pool of patients.

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ERIC-PCR PROFILING, GENETIC ANALYSIS, COLICINOGENY AND LYSOGENY TESTS OF FECAL *ESCHERICHIA COLI* ISOLATES FROM WOMEN WITH GESTATIONAL DIABETES

Luka Predojević[1], Urška Skamen[1], Bernarda Strojin[1], Darja Žgur Bertok[1], Marjanca Starčič Erjavec[1]

[1]Biotechnical faculty, Department of Biology, Slovenia

Pregnancy can cause different kinds of physiological changes, more specifically metabolic changes as well as weight gain, which in turn affect the gut microbiota [1]. Hormonal changes can act as a trigger for conditions like gestational diabetes. Hormonally induced gestational diabetes is caused by proteins synthesized by the placenta, which can increase the level of glucose in the blood during pregnancy [2]. Previous research highlighted a connection between the pre-pregnancy Body Mass Index of women and the development of gestational diabetes during pregnancy [3]. We focused our research on determining a possible correlation between the genetic diversity of facultative anaerobic bacterium *Escherichia coli* (*E. coli*) and the participants weight at the start of their pregnancy in women with gestational diabetes. Participants in this research were divided into two groups according to their initial body weight, a group of participants with normal weight and an overweight group. *E. coli* strains were isolated from fecal samples of participants. The genetic diversity of *E. coli* isolates was determined by ERIC-PCR profiling [4]. PCR was also used to determine the phylogenetic group of *E. coli* [5] and to detect the presence of virulence factor (VF) genes (*sfa*, *papC*, *cnf1*, *hlyA*, *afa*, *iroN* and *ibeA)* as well as certain F-plasmid genes (RepFIA, RepFIB and *traJ*). The strains were further analyzed for bacteriocin production and presence of lysogenic bacteriophages.
ERIC-PCR revealed that among 146 fecal *E. coli* isolates there were 64 different strains. In the normal weight group 44 % of *E. coli* ERIC-PCR profiles were found to be distinct, while in the overweight group there were 52 % of distinct ERIC-PCR profiles. Colicinogenic strains were found to be more frequent in the normal weight group, while no bacteriophages were detected in neither of the groups. We found that the dominant phylogenetical group among the strains from the normal weight and overweight group were B2 and A, respectively. In both groups the dominant VF gene was *iroN*. Further comparison of the two groups revealed differences in both the presence and the prevalence of various genes. All tested VF genes were detected among strains from the normal weight group with *sfa*, *hlyA* and *iroN* being dominant, while in the overweight group genes *sfa*, *cnf1* and *afa* were not found. Region *tra (traJ*) was detected in both groups, while regions RepFIA and RepFIB were detected only in the normal weight group. *E. coli* isolates from the normal weight group were found to be more colicinogenic and belonging mostly to the B phylogenetic group. In conclusion, *E. coli* strains from the normal weight group of participants and *E. coli* strains from the overweight group showed some differences, however to be able to draw final conclusions more strains are planned to be investigated in the future.

WOMEN WITH GESTATIONAL DIABETES: ANALYSIS OF THEIR LIFESTYLE AND GUT MICROBIOTA

Ana Munda[1], Leja Bevc[2], Luka Predojević[3], Katja Molan[3], Simon Koren[4], Draženka Pongrac Barlovič[5,6], Marjanca Starčič Erjavec[3]

[1] University of Ljubljana, Faculty of Arts, Department of Psychology, Slovenia
[2]University of Novo mesto, Faculty of Health Sciences, Department of Nursing, Slovenia
[3]University of Ljubljana, Biotechnical Faculty, Department of Biology, Slovenia
[4]Omega d.o.o., Dolinškova 8, Slovenia
[5]University Medical Centre, Department of Endocrinology, Diabetes and Metabolis Diseases, Slovenia
[6]University of Ljubljana, Medical Faculty, Slovenia

Background: Gestational diabetes mellitus (GDM) is defined as glucose intolerance during pregnancy[2]. It is associated with adverse foetal outcomes as well as with the increased future risk for type 2 diabetes[1]. The aim of our pilot study was to analyze the composition of bacterial gut microbiota in women with GDM. Special emphasis was also placed to record detailed phenotypical data for women enrolled in the study and to investigate the differences in lifestyle among women with GDM prior to conception and during pregnancy.
Methods: 54 women with GDM visiting the diabetes out-patient clinic at University Medical Centre Ljubljana were enrolled into our longitudinal study. Women's Lifestyle was assessed using two custom made questionnaires, focusing on lifestyle before and during pregnancy, based on the National Institute of Public Health principles of healthy eating behavior. The composition of fecal microbiota was determined by sequencing seven different variable regions of the 16S rRNA gene. DNA for the metagenomics analysis was isolated from fecal samples. Then a barcoded amplicon library with the Ion 16S Metagenomics Kit was constructed, library analysis using the LabChip GX instrument was performed, followed by template preparation, enrichment and sequencing on the Ion Torrent next generation sequencing (NGS) PGM platform. Advanced analysis of metagenomic data was completed using the Ion Reporter software.
Results: Scores on the scale of healthy eating behavior were significantly higher during pregnancy (M = 46.28; SD = 6.63) than in pre-pregnancy (M = 54.11; SD = 4.53), $t(35) = 8.834$; $p < 0.001$. A Wilcoxon Signed-ranks test indicated that during pregnancy women with GDM had chosen healthier carbohydrates (Z = 17.5; $p < 0.001$), ate more meals per day (Z = 7.0; $p < 0.001$), ate fried food less often (Z = 40.0; p = 0.019), which is all important for positive regulation of GDM. Composition of bacterial fecal microbiota was successfully determined for all selected samples. Briefly, with a cut off of at least 10 reads passing all quality filters, 93 different families, 87 different genera and 169 different species were reported.
Conclusion: Based on the success of this preliminary study, further sequencing and analysis of larger number of collected samples is ongoing.

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ISOLATION AND QUANTIFICATION OF INDOLE AND SHORT-CHAIN FATTY ACIDS FROM STOOL SAMPLES OF PREGNANT WOMEN WITH GESTATIONAL DIABETES

Marija Trajkova[1], Maja Zugan[1], Katja Molan[1], Matevž Likar[1], Matej Skočaj[1], Darja Žgur-Bertok[1]

[1] University of Ljubljana, Biotechnical Faculty, Department of Biology, Slovenia

ABSTRACT

Indole and short chain fatty acids (SCFA) are secondary metabolites produced by bacteria in the process of tryptophan conversion and fermentation of carbohydrates, respectively. Both indole and SCFA impact host metabolism as well as the gut microbiota. Indole plays an important role in regulation of bacterial biofilm formulation, virulence, sporulation and regulation of numerous bacterial physiological processes. Indole is also a signalling molecule, with which, the gut microbiota communicates with host enteroendocrine L cells influencing host metabolism. Indole modulates the secretion of incretin, a gut hormone, involved in regulation of blood glucose concentrations .

Short chain fatty acids not only serve as an energy source for colonocytes, but also alter the metabolism of fatty acids and hormone production, consequently preventing obesitiy and insulin resistance. Since SCFA and indole are produced in the human colon, they can be easily detected in feces.

The isolation of indole and SCFA from human feces consists of sample homogenization, which must be stored at -20°C using ethanol, centrifugation and collection of the supernatant. The latter is subsequently processed separately depending on wether it is used for detection of indole or SCFA. The concentration of indole was determined using the Kovacs test. Indole produces a red colour, which was spectrophotometrically measured at 550 nm. We also used six known concentrations of indole, to construct a standard curve. We measured the concentration of indole in clinical samples by comparing their absorbance value with those obtained from the measurement curve. The measured concentrations of indole from fecal samples of pregnant women with gestational diabetes (GDM) varied from 0.21 mM to 0.79 mM. As a reference, indole concentrations in fecal samples from healthy individuals is between 0.30 mM and 6.64 mM.

Short chain fatty acids are easily detected using high performance liquid chromatography (HPLC). Prior to sample injection into the HPLC system, samples must be prepared using phosphoric acid and ether, concentrated by eliminating ether using nitrogen gas, and dissolved in methanol. An alternative method, titration, was also used to determine the concentration of fecal SCFA from a healthy individual. The concentrations of SCFA in healthy adults vary from 10 to 100 mmol/L thus, our measured value 22 mmol/L is within the expected range. The profiles of SCFA acquired by HPLC show significant differences among our samples from pregnant women with GDM. The three SCFA, butyrate, propionate and acetate, were most abundant, although isobutyrate, valeric acid and other were also detected.

Transplantation of gut microbiota and use of appropriate probiotics as precautionary measures could exert positive effects on human metabolism.

Key words: feces, indole, short chain fatty acids, HPLC, spectrophotometry

AN ATTEMPT TO ASSESS THE SPREAD OF ANTIBIOTIC RESISTANCE GENES VIA HGT IN FECES OF PREGNANT WOMAN WITH GESTATIONAL DIABETES: A PILOT STUDY

Urša Miklavčič[1], Neža Pajek Arambašić[1], Katja Molan[1] and Jerneja Ambrožič Avguštin[1]

[1] University of Ljubljana, Biotechnical Faculty, Department of Biology, Slovenia

Human gut microbiome research has been an actively developing area for the last decade, revealing new insights into the composition of gut-associated microbial communities in healthy and disease states, interactions of gut microbiota with the central nervous system and disturbance of composition and function of the microbiota due to antimicrobials, thus triggering dysbiosis and the emergence of antimicrobial resistant bacteria/genes. The high density of microorganisms within feces facilitates transfer of antimicrobial resistance genes (ARG) to residual (commensal) and transient (potential pathogenic) bacteria via mechanisms of horizontal gene transfer (HGT): transformation, transduction and conjugation. The aim of our study was to determine the possibilities and conditions to study HGT of ARG, mediated by phages and small plasmids in human feces from pregnant woman with gestational diabetes. Metagenomic DNA (MG-DNA) was isolated from 200 mg feces using the PSP® Spin Stool DNA Kit (Stratec), quantified (NanoDrop) and transformed in competent *Escherichia coli* strain DH5 cells by electroporation and heat shock. Transformants were selected on LB agar plates supplemented with ampicillin, cefotaxime, tetracycline, trimethoprim, ciprofloxacin and imipenem. In additional experiments, 10^7 cells of *E. coli* strain DH5 with plasmid pUC19 Amp^R, were added to the feces prior to isolation of MG-DNA, which was subsequently used for transformation. GeneJET Plasmid Miniprep Kit was used for the isolation of plasmid DNA from transformants. Phage lysates were prepared from feces employing several successive treatments with chloroform and subsequently used for infection of *E. coli* strain J53Az^R, DH5 and HB101Str^R. Transductants were selected on LB agar plates with the same antimicrobials as used for transformation and nalidixic acid. UV light and elevated temperature were used for the induction of the lytic cycle in antimicrobial resistant transductants. Resistance determinants were confirmed by PCR using primers specific for resistance gene groups CTX, SHV, TEM, OXA and PMQR-Qnr. Since none of the tested ARG could be transferred by conjugation of MG-DNA, even from pregnant woman who received antimicrobials, we added strain DH5 (pUC19) to feces, prior to the isolation of MG-DNA. Transformants with pUC19 (frequency of transformation $2{,}5 \times 10^{-8}$, efficiency 5×10^2 cfu/mgr DNA) could be selected only when 10^7 cells of *E. coli* were added to feces, suggesting, that plasmids from bacteria present in feces at lower number could probably not be isolated this way. Additionally, transformation of plasmid DNA from MG-DNA is reduced due to the "noise" of linear MG-DNA. Tranduction experiments yielded several ampicillin (confirmed TEM allele), nalidixic acid and one trimethoprim resistant transductants, probably due to general transduction, since none of them produced phages, after inducing lytic cycle with UV and elevated temperature. Furthermore, transductants were not detected in *recA* deficient strains. Therefore we conclude, that in our pilot study phages were important for HGT of ARG, mainly chromosomal QRDR regions mediating nalidixic acid resistance.

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