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in collaboration with
SLOVENIAN SOCIETY OF HUMAN GENETICS

4th COLLOQUIUM OF GENETICS

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National Institute of Biology
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OPENING LECTURE

Private link between signal and response in bacterial communication stabilizes their cooperative behavior

Ines Mandič Mulec, Anna Oslizlo, Polonca Stefanic, Iztok Dogsa

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Bacteria communicate using freely diffusible signals, which are constitutively secreted during growth. This process is known as quorum sensing (QS) whereby QS signals, after reaching threshold concentration, activate their cognate receptors and induce coordinated response across many cells. As QS is considered a cooperative behavior it is vulnerable to cheating by QS mutants. We addressed the stability of QS by using the ComQXPA QS system of gram positive bacterium *Bacillus subtilis*. In this model the ComQXPA QS coordinates synthesis of the lipopeptide antibiotic surfactin and induction of genetic competence for transformation. By exploring relationships between signal and response in quorum sensing we find that *B. subtilis* mutants deficient in the QS signal (QSS-) become overly responsive to the signal produced by the QS+. This lowers their fitness and prevents them to invade the QS+ population. However, under selective conditions that require DNA exchange signal deficiency transiently brings advantage to the overly responsive QSS- mutant. Under selection pressure the QSS- mutant therefore acts as a hypercheater. The different manifestations of the private link between signal and response, its importance for stability of cooperation during QS and the role of surfactin in the outcome of these interactions will be discussed.

ABSTRACTS of LECTURES

BIOTECHNOLOGY & GENE–ENVIRONMENT INTERACTION

Tanja Guček

Development of new detection methods for identification of causal agents of hop stunt disease in Slovenia

Špela Kos

Differential gene delivery to skin or subcutaneous tissue by varying the gene electrotransfer protocol

Urša Lampreht

Assessment of gene electrotransfer efficiency at different voltages in canine malignant melanoma cells

Nejc Rački

Droplet digital PCR for detection and absolute quantification of waterborne viruses

Progar Vasja

De novo assembly and annotation of *verticillium*-challenged hop transcriptome from short read data

Blažka Smiljanić

Comparitive stress response of two mussel species (*M.edulis* and *M.galloprovincialis*) in response to temperature change

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Abstract

Development of new detection methods for identification of causal agents of hop stunt disease in Slovenia

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Hop stunt disease is an aggressive disease on hop (*Humulus lupulus*) caused by hop stunt viroid (HSVd)[1]. The disease was first discovered in 1940 in Japan and later reported from South Korea, USA, China and, in 2007, also from Slovenia[2,3]. Hop stunt disease spreads rapidly and causes plant stunting, leaf curling, size reduction of hop cones, bine cracking, shortening of the internodes and dry root rot. In Slovenia, a new form of hop stunt disease was found that has more severe plant symptoms, with a shorter incubation period. In addition to HSVd in symptomatic plants, hop latent viroid (HLVd) and a newly discovered citrus viroid were detected. The presence of citrus viroid indicates that this viroid may be the main reason for the new form of hop stunt disease, although detailed etiological studies must be done in the future. The simultaneous presence of several viroids in diseased plants demonstrates the complex nature of this disease; it is therefore necessary to develop reliable diagnostic techniques that will enable accurate disease detection and monitoring of viroids. Various methods are used for the detection of viroids, such as biological testing, molecular hybridization, polyacrylamide gel electrophoresis (PAGE), reverse transcription polymerase chain reaction (RT-PCR), multiplex RT-PCR (mRT-PCR), RT-PCR coupled with hybridization, reverse transcription-loop-mediated isothermal amplification (RT-LAMP) and real time RT-PCR (RT-qPCR) [4]. The most commonly used methods for the detection of hop viroids are RT-PCR and hybridization[5]. In the case of hop stunt disease, the presence of several viroids in a single sample represents a major diagnostics cost, indicating the need for detection methods that are more sensitive and allow simultaneous detection of all viroids, such as mRT-PCR and mRT-qPCR.

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Abstract

Differential gene delivery to skin or subcutaneous tissue by varying the gene electrotransfer protocol

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Gene electrotransfer is an efficient and safe non-viral method for transfecting target cells with selected genes[1]. This technique has become an increasingly popular method to enhance *in vivo* DNA delivery for both, gene therapy applications as well as vaccinaton[2]. One of the interesting targets for gene transfection and vaccination is a skin, due to its easy accessibility for treatment and immunological properties. Since the skin is very complex tissue that consists of different cell types, each cell type can be targeted and transfected by specific gene electrotransfer protocol and other experimental conditions[3]. Therefore, the aim of our study was to control the depth and duration of transfection by gene electrotransfer into the skin or subcutaneous tissue by varying the electric pulse parameters. For this purpose mice were treated with high or low electric pulses after the injection of plasmid DNA encoding for the red fluorescent reporter protein (DsRed). To determine the transfection efficiency, expression of DsRed protein was observed using fluorescence microscopy. Our results indicate that high voltage pulses promote transfection in the skin (epidermis and dermis), while low voltage pulses affect deeper subcutaneous tissue (panniculus carnosus). Differential transfection of skin layers under different electric conditions has a great potential to adjust the duration of gene therapy effect and differentiate between the local and systemic effects.

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Abstract

Assessment of gene electrotransfer efficiency at different voltages in canine malignant melanoma cells

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The use of murine cancer models in translational medicine is limited due to differences in size and physiology as well as variations in the homology of targets between mice and humans[1]. Most of canine malignant melanoma is homologous to human melanoma types and for these reasons spontaneous malignant melanomas in dogs revealed to be an excellent model for human melanomas[2]. Therefore, studies using canine tumor cells and spontaneous tumors are becoming more and more valued and represent an important translational bridge to human medicine. Due to lack of gene electrotransfer studies in canine cell lines we designed our study to determine the transfection efficiency of plasmid DNA encoding green fluorescence protein (GFP) at different voltage in canine melanoma cell lines. Two canine malignant melanoma cell lines (CMeC-1 and CMeC-2[3]) and one human skin malignant melanoma (SKMel28) cell line were transfected with plasmid pEGFP-N1 encoding GFP using electroporation at different voltages that ranged from 450V/cm to 600V/cm. The survival of these cell lines after gene electrotransfer was also determined by cell viability assay Presto Blue. Transfection of CMeC-1 and CMeC-2 with EGFP-N1 plasmid was the highest at 500V/cm and of SKMel28 at 450V/cm. The survival was overall better at 450V/cm in all three cell lines but due to pronounced transfection efficiency, the 500V/cm voltage was selected in CMeC-1 and CMeC-2 cell lines for further experiments. Our results demonstrated higher transfection and better survival of CMeC-1 cells compared to CMeC-2 cells. Therefore, CMeC-1 cell line seems more suitable for further research for in vitro and in vivo gene therapy studies using electrotransfer.

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Abstract

Droplet digital PCR for detection and absolute quantification of waterborne viruses

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Ground waters represent a major transportation vector for viruses which are resistant to environmental degradation. The main source of such viruses is wastewater. Because traditional wastewater treatments do not remove them efficiently, infectious viruses are released daily into environmental waters[1] and can find their way into irrigation or even potable water sources. The infective doses of these viruses can be extremely low, down to only a few ingested virus particles. Therefore detection methods for their monitoring need to be particularly sensitive and repeatable at low concentrations. With increasing number of regulatory bodies shifting towards the implementation of quantitative microbial risk assessment (QMRA)[2] to determine the acceptable water contamination level, not just precise detection but also absolute quantification is gaining importance. Real-time quantitative PCR represents a golden standard for molecular virus detection and quantification however; its quantitative use is linked to standardized reference materials. Its sensitivity to inhibitors can lead to lower repeatability and accuracy of quantification. Droplet digital PCR has been proposed as a method to overcome these drawbacks. In our research we were the first to successfully implement the use of a one step reverse-transcription droplet digital PCR (RT-ddPCR) for absolute quantification of an RNA virus[3]. We demonstrated that RT-ddPCR shows higher tolerance to inhibitors from environmental and plant samples. RT-ddPCR proved to be more precise and more tolerant to inhibitory substances than the benchmarking RT-qPCR, and needs no standard curve making it a method of choice for QMRA.

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Abstract

De novo assembly and annotation of *verticillium*-challenged hop transcriptome from short read data

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Hop (*Humulus lupulus* L.) is an important agricultural plant, mainly valued for its use as an essential ingredient of beer. One of the most threatening diseases in hop production is Verticillium wilt, caused by the soil-borne fungus *Verticillium albo-atrum*. The severity of the disease varies, depending on the resistance exhibited by the particular hop cultivar.

In order to examine the resistance mechanisms, the interactions of resistant and susceptible cultivars infected with *V. albo-atrum* were analyzed in a previous experiment by differential display methods and RT-qPCR[1]. To further elucidate these mechanisms, we conducted another extensive inoculation experiment on the same two cultivars, under various conditions, this time using deep transcriptome sequencing (RNA-Seq).

RNA-Seq produces very short reads and a plausible transcriptome assembly is essential for downstream differential expression analysis. Since the hop genome has not yet been sequenced, we had to perform *de novo* assembly.

In addition to alternative splice forms and the heterozygosity of alleles, in plants, successful assembly is complicated by extensive gene duplication and polyploidy[2]. Furthermore, it is difficult to assess the quality of an assembly - standard metrics based on contig number and lengths might not be the best representation thereof[3].

We used a previous local version of the assembled hop transcriptome as a model and, in order to include cultivar-specific and disease-induced transcripts, updated it with an assembly of reads that could not be mapped to the model. We evaluated several approaches to assembly and performed similarity-based annotation of *Verticillium* challenged hop transcriptome, the results of which will be presented.

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Abstract

Comparitive stress response of two mussel species (*M.edulis* and *M.galloprovincialis*) in response to temperature change

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Mytilus edulis and *Mytilus galloprovincialis* are two species of blue mussel and water temperature is the most important factor influencing their distribution. *Mytilus edulis* inhabits temperate waters of North Atlantic Ocean, while *Mytilus galloprovincialis* is abundant in the Mediterranean Sea and it also extends to the Southwest Coast of the United Kingdom, where the two species can hybridize. Although species are phylogenetically closely related they have different physiological tolerances to environmental conditions such as temperature. If temperature rises beyond these limits it causes stress, which could make the animals more susceptible to contaminant exposure and may also affect the performance and interpretation of commonly used ecotoxicological endpoints in biomarker assays. Mussels were collected from the North Sea and Northern Adriatic Sea and exposed to different temperature ranges (11°C, 17°C, 20°C and 25°C) for 7 days. Their response to stress was assessed with Comet assay, NRR assay, TBARS assay and SOD assay. These assays show DNA damage, lysosomal membrane permeability and oxidative stress, respectively. Higher temperatures of 20°C and 25°C had an effect on DNA and structural integrity of lysosomes. Damage to DNA and lysosome membrane integrity were higher in *M. galloprovincialis* than in *M. edulis*. However there were no significant effects observed for TBARS and SOD assay. This could be the result of *M. galloprovincialis* already existing at the higher end of its temperature tolerance limit and indicates the potential impact of climate change on ecotoxicological studies.

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ABSTRACTS of LECTURES

MOLECULAR BASIS OF DISEASES & POPULATION GENETICS

Carina E.P. Kozmus

A 1p36 polymorphism is associated with atopic childhood asthma onset, severity, treatment outcome with inhaled corticosteroids, and with the expression of the cannabinoid receptor 2 (*CNR2*) gene

Maša Bosnjak

Gene electrotransfer of Plasmid AMEP as integrin targeted therapy for murine melanoma tumors

Alenka Matjašič

Glioma subtype-associated lncRNAs and lncRNA-related miRNAs expression patterns

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Plasmid DNA encoding shRNA against endoglin has comparable antitumor effect when using tissue specific or constitutive promoter

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Amyotrophic lateral sclerosis in Slovenian population: genetics

Lucija Raspor Dall'Olio

Phylogenetic characterizaton of *Symbiodinium sp*. from scyphozoan hosts

Anja Plemenitaš

Genetic influences and alcohol dependence in Slovenian population

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Abstract

Polymorphism on chromosome 1p36 is associated with atopic childhood asthma onset, severity, treatment outcome with inhaled corticosteroids, and with the expression of the cannabinoid receptor 2 (*CNR2*) gene

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Asthma pathogenesis is currently understood through the interaction of several genes and environmental influences. It is believed that endocannabinoids act as native modulators of the immune system, probably through activation of the cannabinoid receptors. Recent expression quantitative trait loci studies linked single nucleotide polymorphisms (SNPs) on chromosome 1p36 with *CNR2* gene expression. We analyzed rs4237 association with childhood asthma, and the effect of rs4237 on the response to inhaled corticosteroids (ICS) treatment and *CNR2* gene and protein expression. We studied a case-control cohort of 229 children mild/moderate persistent asthma (150 atopic, 79 non-atopic, 13 undetermined atopy), and 271 controls. Blood samples were collected before treatment and 72 matching samples 4-6 weeks after treatment with ICS. According to recessive model of genetic association the frequency of CC genotype in atopic asthmatics was lower than in controls ($p=0.0284$). Forced expiratory volume in 1 second (FEV1) was higher in atopic asthmatics with CC genotype compared to those with CT or TT ($p=0.0155$). A strong positive correlation between the *CNR2* RNA and protein levels was found ($p<0.0001$). Median relative expression of *CNR2* in asthmatics with TT genotype was higher compared to those with CT or CC ($p=0.0041$). FEV1 increased significantly after ICS treatment in atopic asthmatics with TT genotype, compared to those with CT or CC homozygotes ($p=0.034$). Our results suggest rs4237 is associated with asthma onset, with asthma severity, *CNR2* gene expression and with inhaled corticosteroids treatment response in children with atopic asthma, and suggest the involvement of the endocannabinoid system in asthma.

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Abstract

Genetic influences and alcohol dependence in Slovenian population

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Studies have convincingly demonstrated that genes play an important role in the development of alcohol dependence [1]. Several genes are known to contribute to this hereditability, including the genes involved in ethanol metabolism [2]. Genes involved in the serotonin pathway may determine severity of and susceptibility for alcohol dependence[3]. The present study explored whether specific *polymorphisms* in alcohol metabolic pathway and the serotonin pathway could be associated with alcohol dependence.

The cohort comprised 101 currently and 100 formerly alcohol-dependent subjects, as well as 97 healthy blood donors. AUDIT questionnaire was employed. Subjects were genotyped for *CYP2E1* c.-1053C>T, *CAT* c.-262C>T, bi-and tri-allelic *HTTLPR*, *5-HTR1A* rs6295 and *5-HTR1B* rs13212041.

Statistically significant differences in the distribution of *CAT* c.-262C>T genotypes and alleles were observed among the three investigated groups. We observed a higher frequency of *CAT* -262T allele in alcohol-dependent subjects (OR = 1.74, 95% CI = 1.164 - 2.610). Among currently dependent patients, carriers of the *CAT* -262T allele had higher scores on AUDIT questionnaire. Statistic differences in bi-and tri-allelic *5-HTTLPR* genotypes distribution were observed among the three groups investigated (p = 0.008 and p = 0.023 respectively), however no gene-dose effect was observed. The severity of alcohol problems was higher in currently alcohol-dependent subjects with *5-HTTLPR* LL (p = 0.039) and L'L' genotype (p = 0.027).

Our findings suggest that the *CAT* c.-262C>T genetic polymorphism influences the susceptibility to alcohol dependence and severity of alcohol dependence, while bi-and tri-allelic *5-HTTLPR* has some effects on the severity of alcohol dependence.

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Abstract

Gene electrotransfer of Plasmid AMEP as integrin targeted therapy for murine melanoma tumors

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Plasmid AMEP codes for AMEP peptide (**A**ntiangiogenic **ME**targidin **P**eptide), which binds specific integrins that are overexpressed on tumor and activated endothelial cells. Thus, it has dual effect; antiangiogenic and direct antitumor.[1,2] The aim of the study was to inhibit angiogenesis and tumor progression after gene therapy with Plasmid AMEP. As a delivery method, gene electrotransfer (GET) was used. Eight square wave, high voltage pulses (voltage-to-distance ratio 600 V/cm), with duration of 5 ms and frequency 1Hz were used to enable entering of plasmid DNA into the cells. Antitumor effectiveness was determined by tumor growth delay assay and histological staining for tumor cell proliferation, necrosis and apoptosis. Antiangiogenic potential of the therapy was determined separately by histological staining of tumor sections with antibodies against endothelial marker CD31 and intravital microscopy, where the formation and morphology of developing vessels were observed in tumors implanted in dorsal window chamber in mice.

The study showed significant antitumor effectiveness of GET of Plasmid AMEP, resulting in tumor growth delay, which did not correlate to AMEP mRNA expression levels in tumors after GET, but to the quantity of the integrins on the tumor cells. Additionally, histological staining of tumor sections for tumor necrosis, apoptosis and proliferating cells confirmed antitumor effectiveness.

Antiangiogenic effectiveness was demonstrated in dorsal window chamber model, by the inhibition of tumor blood vessels formation in the treated tumors. In accordance with these results, histological analysis of CD31 vessels staining showed a reduced number of vessels in treated tumors.

This study showed that GET of Plasmid AMEP is effective therapy for treatment of murine melanoma tumors, where the quantity of integrins on melanoma cells is a crucial factor for its effectiveness.

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Abstract

Glioma subtype-associated lncRNAs and lncRNA-related miRNAs expression patterns

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Gliomas are the commonest and highly malignant primary brain tumors. Their classification is based upon malignancy grade and histological characteristics, however the correct diagnosis that relies solely on histopathological characteristics might be difficult and inadequate, especially with cases lacking typical features. Glioma subtypes have distinct molecular features and expression analyses could uncover biomarkers for deciphering glioma subtypes. Our purpose was to identify lncRNAs that might play role in gliomagenesis and investigate association of differential expression profiles with different glioma subtypes, as well as association with their co-regulated/-expressed miRNAs.

We included sixty-four patients and used qPCR approach for determining differentially expressed lncRNAs using LncRNA array profiler on a smaller cohort of pathohistologically evaluated glioma samples normalized to human brain reference RNA. A subset of differential lncRNAs and lncRNA-related miRNAs were further validated by qPCR approach on a biger cohort of glioma samples.

LncRNA profiling revealed a total of 74 among 90 analysed lncRNAs to be widely expressed and statistical tests identified a subset of 10 significantly differentially expressed lncRNAs. Further analyses of 7 dysregulated lncRNAs (*7SL*, *EGOA*, *HOTAIR*, *JPX*, *MEG3*, *RNCR3* and *ZNFX1-AS1*) have showed statistically significant expression differences implicating distinctive expression profiles in glioma subtypes. We observed a correlation in expression of *miR-124a* and *RNCR3*, *miR-196a* and *HOTAIR* and *miR-770* and *MEG3*.

Our findings support the concept of lncRNAs and miRNAs as molecular biomarkers by determining distinctive subtype-related lncRNA/miRNA expression patterns, suggesting an important role of ncRNAs in gliomagenesis and implicate the potential use of lncRNAs in histological classification.

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Abstract

Phylogenetic characterization of *Symbiodinium sp*. from scyphozoan hosts

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The dinoflagellate species *Symbiodinium sp*. is divided into several clades (designated from A to I) by using genetic markers (18S rDNA, 28S rDNA, ITS, ITS2, cp23S rDNA). Among them clade A was found many times in different scyphozoan hosts (e.g. *Cassiopea sp.*, *Mastigias* sp., *Cotylorhiza tuberculata* and *Linuche unguiculata* (LaJeunesse 2001, Santos 2002, Yamashita 2012).

In present study, Symbiodinium sp. was isolated from *Cotylorhiza tuberculata*, *Phyllorhiza punctata* and *Cassiopea xamachana*. Their identity and genetic diversity at intra and inter population level was investigated with nuclear 28S rDNA and ITS2 rDNA markers. 28S rDNA was amplified by primer pair 28Sfor and 28Srev (Zardoya 1995), ITS2 region was amplified by primer pair ITSintfor2 and ITS2rev (LaJeunesse 2000). The length of 28S alleles were 630 bp and ITS2 alleles were from 330 to 360 bp. Phylogenetic analysis revealed that *Symbidinium sp.* cells isolated from previously mentioned species belong to clade A, B and C (results from both markers are mostly congruent). Two clades (A and B) were found in *C. tuberculata,* clade A was found in *P. puctata*, while *Cassiopea xamachana* contain clade C and B. According to result we assume that: individual scyphozoan host contain one type of *Symbiodinium* clade, while different individuals of the same scyphozoan species can contain different clades. Moreover, distribution of clades A and B is not restricted to particular region or to scyphozoan host. This was demonstrated by *C. tuberculata* from Mljet Lake contain mostly clade A, but individuals infected with clade B are also found, moreover *C. tuberculata* from Mar Menor lagoon contain only clade B. It is still open question whether infection of host is related to abundance of certain clade in the particular environment.

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Abstract

Plasmid DNA encoding shRNA against endoglin has comparable antitumor effect when using tissue specific or constitutive promoter

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Antiangiogenic therapies targeting specific endothelial cell markers are promising approaches for the treatment of cancer. One of the targets is endoglin, a Transforming Growth Factor β (TGF-β) coreceptor, involved in cellular proliferation, differentiation and migration [1,2]. We already demonstrated that endoglin silencing with siRNA has antitumor effect [3], where gene electrotransfer is used as delivery method. To prolong the effect, we constructed two plasmid DNA (pDNA) encoding shRNA against endoglin; one with constitutive promoter (CON) and the other with tissue specific promoter for endothelin-1 (TS) to increase specificity for endothelial cells, in order to compare theirs effectiveness. *In vitro*, we confirmed tissue specificity of TS plasmid by measuring transfection efficiency in specific cell lines using flow cytometer and demonstrated the same antiangiogenic potential on tube formation of endothelial cells as if using CON plasmid. *In vivo*, BALB/c mice bearing non-endoglin expressing TS/A murine mammary carcinoma, were divided in two groups with different tumor sizes and vascularization statuses, and subjected to the same therapy protocol. Based on tumor growth curves, the therapy with either of both therapeutic plasmids (TS and CON) was efficient in both tumor groups, although the effect was more pronounced when applied on smaller tumors in avascular phase. In the therapeutic groups, additional histological analysis demonstrated significant increase of necrosis and decrease of number of blood vessels. In conclusion, both gene electrotransfers of pDNAs against endoglin had good and comparable antitumor effect, confirming the future potential of using TS plasmid, as it proves to be more specific and safer than CON plasmid.

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Abstract

Amyotrophic lateral sclerosis in Slovenian population: genetics

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Amyotrophic lateral sclerosis (ALS) is a complex neurodegenerative disease[1, 2] characterised by progressive degeneration and loss of upper motor neurons in the cerebral cortex and lower motor neurons in brainstem and spinal cord[2] leading to death due to respiratory failure within 2-5 years from onset[3]. The most common genes involved in the disease process are *SOD1*[4], *FUS*[5, 6], *TARDBP*[7-9] and *C9ORF72*[10, 11]. For the research purposes blood samples from 94 Slovenian ALS patients were collected. Genotyping of *SOD1*, *FUS* and *TARDBP* genes by PCR amplification of exons of interest followed by Sanger sequencing revealed 4 changes in the coding DNA sequence. In *SOD1* gene two mutations (p.Val14Met and p.Gly93Cys) were detected. Sequencing of *FUS* and *TARDBP* genes did not reveal any amino acid changes. Although two substitutions were detected, p.Arg522Arg (G>A) in *FUS* and p.Leu330Leu (A>G) in *TARDBP* the latter was not previously described. In case of *C9ORF72* repeat-primed PCR was performed followed by fragment length analysis on ABI310 Genetic Analyzer. Results were analyzed using Gene Scan software. Expansion of hexanucleotide repeats (GGGGCC) in the first intron of *C9ORF72* gene was detected in 5 patients. This study represents first genetic analysis of Slovenian ALS patients. Our findings are in concordance with other studies provided on European ALS patients[12]. Of these four analyzed genes repeat expansion in *C9ORF72* remains the most common cause of ALS although for the majority of cases the main causes are yet to be revealed.

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POSTERS

ABSTRACTS of POSTERS

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Efficiency of an antimicrobial conjugation-based system against selected pathogens

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Extra-intestinal pathogenic *Escherichia coli* strains with different genetic backgrounds elicit different immune responses

Tanja Zadražnik, Wolfgang Egge-Jacobsen, Jelka Šuštar-Vozlič

Proteomics analysis of drought stress: glycoproteins in common bean

Mateja Zupin, Marko Maras, Vladimir Meglič

Development of genetic map of common bean (Phaseolus vulgaris l.) for quantitative trait loci detection involved in response to drought

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Abstract

A mouse Atlas of enzyme-coding gene

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A relatively large proportion of the human population still remains lean despite rapid spreading of obesity due to the modern »obesogenic« environment, suggesting genetic resistance to obesity development. Our research identified thiosulfate sulfurtransferase (*Tst*) as the only upregulated adipose-specific gene by fine mapping, gene expression and functional analysis of the ~ 20 lean gene candidates within the genetic interval *Fob3b2* that confers anti-obesity effects in a polygenic mouse model. We propose that *Tst* is a novel gain-of-function gene in adipocytes that promotes healthy leanness, a proposition by our extensive studies in mice selected for high or low fat content and our collaborators using transgenic models and human genetics approaches. So far, functional roles of TST have been implied in iron-sulfur cluster formation, cyanide detoxification and 5S ribosomal RNA import into mitochondria but the gene has not yet been linked to obesity/leanness control. To enable further studies of *Tst* roles in other biological processes such as obesity and diabetes, a mouse Atlas of enzyme-coding gene was generated. Integrated data in the atlas were derived from various databases and experiments as well as from bioinformatics predictions. For a more comprehensive knowledge about *Tst* gene and identification of its direct or indirect target genes, functional polymorphisms, association studies, transcription factors, chromatin modifications, regulatory features and miRNA binding sites were integrated into the *Tst* regulatory atlas. This atlas will present a valuable tool for planning efficient further experimental validations and analyses of this causal leanness gene for basic research questions as well as for potential therapeutic developments.

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Abstract

Efficiency of an antimicrobial conjugation-based system against selected pathogens

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A clear imperative exists to develop new alternative antimicrobial agents and treatments to respond to the emerging threat of infections with multiple-drug resistant bacteria. A new concept of bacterial conjugation-based technologies has been proposed[1], which exploits plasmid biology to combat the aforementioned problem. The F plasmid is the best characterised conjugative plasmid of the bacterial species *Escherichia coli*. Since its discovery, it has served as an archetype for studies of bacterial conjugation. Along the lines of bacterial conjugation-based technologies, we have developed an F plasmid based system[2] for the delivery of toxic genes that destroy pathogenic *E. coli* strains as well as strains belonging to closely related genera. As the host strain of the conjugative plasmid, we have employed the probiotic *E. coli* strain Nissle 1917, while for the toxic gene harboured on the plasmid, we have used the colicin E7 synthesis gene, encoding a non-specific DNase. The aim of our study was to determine the efficiency of our system as an alternative antimicrobial agent against uropathogenic strains of *E. coli*, Shiga toxin type II producing *E. coli*, and *Salmonella sp.* The results of our study indicate that the used conjugative plasmid with the colicin E7 synthesis gene is transferred to recipient strains and that it is indeed an effective antimicrobial agent active against the investigated pathogenic strains. Further studies aimed at characterising the most suitable potential targets against which this antibacterial agent could be applied are planned.

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Article

Extra-intestinal pathogenic *Escherichia coli* strains with different genetic backgrounds elicit different immune responses

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Escherichia coli* (*E. coli*) is a normal inhabitant of the intestinal microbiota of vertebrates, serving as a barrier to colonisation by pathogens in the intestinal tract. On the other hand, some strains of *E. coli* are pathogenic and are important etiological agents of intestinal (intestinal pathogenic *E. coli* – InPEC) as well as extra-intestinal (extra-intestinal pathogenic *E. coli* – ExPEC) infections in humans. While commensal strains of *E. coli* typically harbour none or very few virulence factors, pathogenic *E. coli* strains usually carry multiple virulence factors, enabling them to colonise the host and cause disease. Many virulence-associated genes have been discovered in pathogenic *E. coli* strains. Further it is known, that there is a link between possession of virulence related genes and the phylogenetic group. To determine whether ExPEC strains belonging to different genetic backgrounds provoke different macrophage IL-6 responses, we used two different ExPEC strains, one belonging to the phylogenetic group A and the other to the phylogenetic group B2. The highest IL-6 mRNA expression levels in J774A.1 murine macrophage cells were observed upon stimulation with live bacteria. Furthermore, the B2 group strain possessing a higher virulence score was shown to elicit markedly higher macrophage IL-6 mRNA expression levels, compared to the A group strain with a lower virulence score. Our findings indicate certain differences in eliciting macrophage IL-6 response (measured by IL-6 mRNA expression levels) between pathogenic *E. coli* strains belonging to different phylogenetic groups, with different sets of virulence factors.

INTRODUCTION

Escherichia coli (*E. coli*) is a normal inhabitant of the intestinal microbiota of vertebrates, serving as a barrier to colonisation by pathogens in the intestinal tract[1]. On the other hand, some strains of *E. coli* are pathogenic and are important etiological agents of intestinal (intestinal pathogenic *E. coli* – InPEC) as well as extra-intestinal (extra-intestinal pathogenic *E. coli* – ExPEC) infections in humans. *E. coli* has been implicated in diarrhoea and urinary tract infections (UTI), as well as in skin and soft tissue infections, neonatal meningitis, and septicaemia[2, 3]. While commensal strains of *E. coli* typically harbour none or very few virulence factors, pathogenic strains of *E. coli* usually carry multiple virulence factors, enabling them to colonise the host and cause disease. Many virulence-associated genes in pathogenic *E. coli* strains have been discovered[4].

The innate immune system is the first line of host defence against pathogens. It covers many areas of host defence against pathogenic microbes. One of its key roles is to distinguish between self and non-self or danger, and where possible between self and pathogen. This differentiation is mediated by the interaction of two sets of complementary molecules: pathogen-associated molecular patterns (PAMPs), which are generally conserved among pathogens, and host structures called pattern recognition receptors, which recognise the corresponding PAMPs[5, 6]. PAMPs define many of the properties of host-parasite interaction. They stimulate the innate immune system to produce pro-inflammatory cytokines and play a key role in pathogenesis. The immunodominant PAMP of most Gram-negative bacteria is the lipid A portion of the lipopolysaccharide (LPS)[7-10].

The aim of this study was to determine whether differences exist in macrophage IL-6 responses to ExPEC strains belonging to different genetic backgrounds. We used two ExPEC strains, one belonging to the low virulence phylogenetic group A, and the other belonging to the high virulence phylogenetic group B2[11].

METHODS

Bacterial strains and growth conditions. *E. coli* strains used in this study, DL94 and TA131, were taken from the ExPEC strain collection of the Department of Biology, Biotechnical Faculty, Ljubljana. The relevant features of both used strains are presented in Table 1. Strains were grown in liquid LB Lennox medium (MP Biochemicals) at 37°C with aeration (160 – 180 rpm).

Table 1: Bacterial strains used in this study

***Escherichia coli* strain**	**Relevant features**
DL94	ExPEC isolated from urine of a female patient suffering from a urinary tract infection Phylogenetic group A (determined as in Clermont et al., 2000)[12] Streptomycin sensitive (200 µg/ml)
TA131	ExPEC isolated from a decubitus wound of a female patient Phylogenetic group B2 (determined as in Clermont et al., 2000)[12] Streptomycin sensitive (200 µg/ml)

Phylogenetic grouping and virulence associated gene (VAG) typing. Strains were phylo-typed according to the new phylo-typing method proposed by Clermont et al. in 2013[13]. Strains were VAG-typed for 17 different virulence associated traits: type 1 fimbriae (*fimH*), P-fimbriae (*pap*C, *papG*II, *papG*III), S- and F1C-fimbriae (*sfaDE/focIC*), afimbrial/Dr adhesin (*afaBC*/*draBC*), hemolysin (*hlyA*), cytotoxic necrotizing factor 1 (*cnf1*), uropathogenic specific protein (*usp*), colibactin (*clbA*, *clbQ*), invasin Ibe (*ibeA*), aerobactin (*iucD*), salmochelin (*iroN*), yersiniabactin (*fyuA*), siderophore receptor IreA (*ireA*), group II capsule incl. K1 capsule (*kpsMT*II), increased serum survival (*traT*), suppression of innate immunity – Toll/interleukin-1 receptor domain-containing protein Tcp (*tcpC*), and outer membrane protease T (*ompT*). All PCR reactions were performed on bacterial lysates according to Le Bouguenec et al., 1992[14]. Published PCR-primers and PCR-protocols were used[14-27]. Virulence score was determined by counting possession of the following genes: *fimH*, *papC*, *papG*II, *papG*III, *sfaDE/focIC*, *afaBC/draBC*, *hlyA*, *cnf1*, usp, *clbAQ*, *ibeA*, *iucD*, *iroN*, *fyuA*, *ireA*, *kpsMTII*, *traT*, *tcpC,* and *ompT* (maximum possible virulence score 19).

Cell culture. J774A.1 macrophages were routinely cultured in 25 cm^2 cell culture flasks (Corning) in Dulbecco's Modified Eagle Medium (DMEM) (PAA Laboratories) supplemented with 10 % non-heat inactivated fetal calf serum (FCS) (PAA Laboratories) at 37°C in a 10% CO_2 atmosphere.

Preparation of bacterial strains for macrophage stimulation. Overnight cultures of bacterial strains listed in Table 1 were obtained and optical density at 600 nm was measured. Bacteria were harvested by centrifugation and suspended in Dulbecco's phosphate-buffered saline (dPBS) without Ca^{2+} and Mg^{2+} ions (PAA Laboratories) at a concentration of 2.5×10^9 bacteria per ml. To prepare bacterial lysates, bacteria kept in dPBS were sonicated on ice for 10 minutes at 30% sonicator duty cycle; sufficient bacterial lysis was verified by phase contrast microscopy. Alternatively, streptomycin-treated bacteria were used. Hereto, streptomycin (200 µg/ml) was added to bacteria suspended in dPBS and cells were incubated at 37°C and 160 – 180 rpm for 4 h.

Stimulation with bacterial strains. Prior to stimulation with bacteria, J774A.1 macrophages were grown to 80 – 90% confluence in a 24-well tissue culture plate (Corning) using the same media and growth conditions as described above. J774A.1 macrophages were then stimulated with

sonicated, streptomycin-treated, and live bacteria of *E. coli* strains listed in Table 1. Four µl (corresponding to 1×10^7 sonicated, streptomycin-treated, or live bacterial cells) of either sonicated, streptomycin-treated, or live bacterial cells were added to separate wells with J774A.1 macrophages in DMEM supplemented with 10% non-heat inactivated FCS. Macrophages were incubated at 37°C in a 10 % CO_2 atmosphere for 4 hours. After a 2-hour stimulation, wells containing macrophages with live bacteria were washed twice with dPBS and subsequently incubated for another 2 hours in DMEM supplemented with 10% non-heat inactivated FCS at 37°C in a 10% CO_2 atmosphere. *Neisseria meningitidis* LPS (50 ng/ml) and streptomycin (200 µg/ml) were used as controls in separate wells. Wells with no additional stimulants were used as a negative control. Stimulation was performed twice in duplicates.

RNA isolation from J774A.1 macrophages. RNA was isolated using RNA Bee (AMS Biotechnology) RNA isolation reagent according to manufacturer's instructions. RNA was dissolved in 30 µl of diethyl pyrocarbonate-treated water (DEPC-H_2O) (Life Technologies). DNase I treatment of isolated RNA was performed with DNase I supplied by Invitrogen, following manufacturer's instructions. After DNase I inactivation, RNA was diluted to a final concentration of 10 ng/µl with DEPC-H_2O.

Quantitative reverse-transcription PCR (qRT-PCR). qRT-PCR was performed using "Agilent Brilliant III Ultra-Fast SYBR® Green QRT-PCR Master Mix" kit and "Roche LightCycler® 480 System" following manufacturers' instructions. qRT-PCR was performed for the IL-6 and β-actin genes. The primers used in this study are listed in Table 2. The qRT-PCR program employed was designed according to the manufacturers' instructions. The fold increase in the expression of the IL-6 gene relative to the negative control and normalised to the β-actin gene was calculated using the $2^{-\Delta\Delta C_T}$ method, as described by Livak et al[28].

Table 2: Primer oligonucleotides used in qRT-PCR.

Primer oligonucleotide		Sequence
IL-6	mIL-6 RT F	5'-TTCCATCCAGTTGCCTTCTTG-3'
	mIL-6 RT R	5'-TCATTTCCACGATTTCCCAGAG-3'
β-actin	mActin RT F	5'-TCCTGTGGCATCCACGAAACT-3'
	mActin RT R	5'-GGAGCAATGATCCTGATCTTC-3'

Quantitative reverse transcription PCR was performed with four batches of isolated RNA. Arithmetic means and standard errors of the fold increases of the IL-6 gene expression levels were calculated. Standard errors were plotted as error bars.

RESULTS

Phylogenetic groups and VAGs. As Clermont et al. in 2013 revised their original method of PCR phylo-typing and presented an improved, more specific method revealing also new phylo-groups, both strains used in this study were first retyped according to this new phylo-typing method[13]. Both strains remained in the same phylogenetic groups, DL94 in the phylogenetic group A and TA131 in the phylogenetic group B2, as determined previously (following Clermont et al[12]). The VAG results for both strains are presented in Table 3.

Table 3: Virulence associated genes (VAGs) present in DL94 and TA131.

Virulence trait group	VAG	Strain DL94	Strain TA131
Adhesin/Fimbriae	*fimH*	+	+
	papC	+	–
	*papG*II	+	–
	*papG*III	–	–
	sfaDE/focIC	–	+
	afaBC/draBC	–	–
Toxin	*hlyA*	–	–
	cnf1	–	–
	usp	–	–
	clbAQ	–	–
Invasin	*ibeA*	–	–
Iron up-take system	*iucD*	–	+
	iroN	–	–
	fyuA	+	+
	ireA	–	–
Protectin	*kpsMT*II	–	+
	traT	–	–
	tcpC	–	–
	ompT	–	+
Virulence score		4	6

The presence of a certain gene is marked: +, gene was detected; –, gene was not detected.

Macrophage response. To determine any differences in macrophage IL-6 mRNA expression levels in response to ExPEC strains of different genetic backgrounds, we stimulated the J774A.1 murine macrophage cells with one group A ExPEC strain (DL94) and one group B2 ExPEC strain (TA131). Macrophages stimulated with strain TA131 (Figure 1) displayed the highest IL-6 mRNA expression levels when stimulated with live bacterial cells. Streptomycin-treated bacteria of strain TA131 induced significantly reduced levels of IL-6 mRNA compared to stimulation with live bacteria of strain TA131. Sonicated bacteria of strain TA131 induced the lowest IL-6 mRNA expression levels compared to stimulation with live and streptomycin-treated bacteria of strain TA131. When macrophages were stimulated with strain DL94, the same trend could be observed, although the differences were less marked. The greatest difference in macrophage IL-6 mRNA expression levels was found after stimulation with live bacteria of either strain TA131 or DL94, although strain TA131 induced a significantly higher IL-6 mRNA expression level compared to strain DL94. No significant difference between strains could be observed when macrophages were stimulated with sonicated bacteria. In all cases, the macrophage response to the bacteria was stronger than that induced by stimulation with 50 ng/ml *Neisseria meningitidis* LPS, which was used as a positive control.

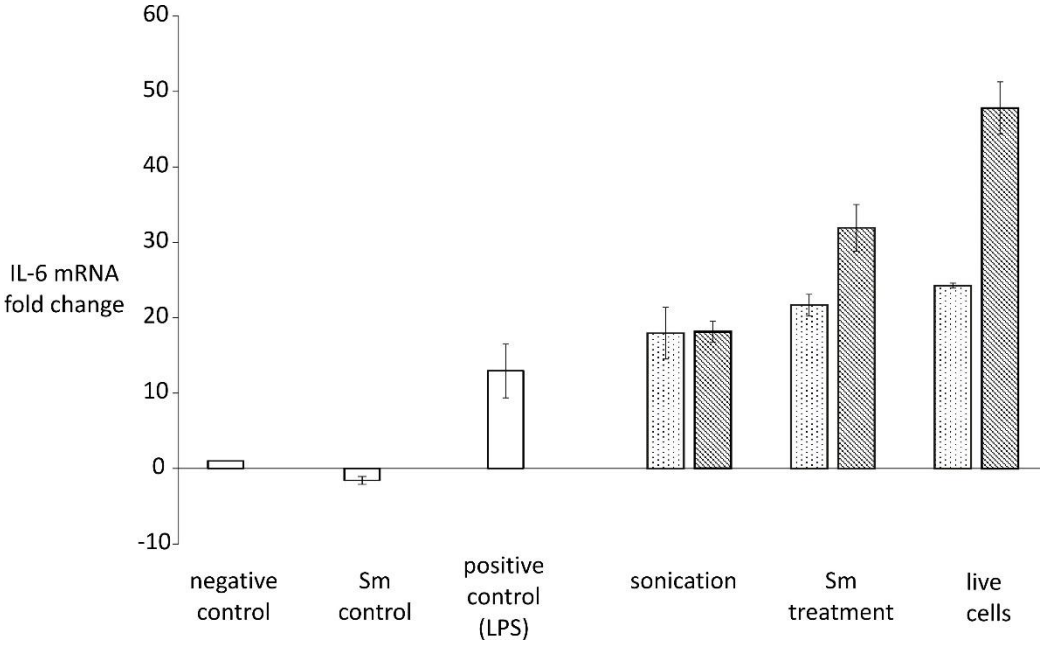

Figure 1: IL-6 mRNA expression levels of macrophages stimulated with strains DL94 (dotted) and TA131 (hatched), calculated relative to the negative control, and normalized to the β-actin gene. Error bars represent standard errors. LPS, lipopolysaccharide; Sm, streptomycin.

DISCUSSION

E. coli strains belonging to phylogenetic group A usually harbour none or very few virulence genes and generally do not cause diseases but are typically commensal strains. *E. coli* strains belonging to phylogenetic group B2, on the other hand, usually possess a greater number of virulence determinants, and are frequently found to cause infections, often with a pronounced host immune response. With these findings in mind, we conducted a preliminary study to determine possible differences in macrophage IL-6 response between *E. coli* strains of the two aforementioned phylogenetic groups. The results show significant differences in macrophage IL-6 responses for strains DL94 and TA131, with strain TA131 eliciting a much higher macrophage IL-6 mRNA expression level compared to strain DL94. It is known that pathogenic bacteria have evolved numerous mechanisms for the modification of the lipid A portion of LPS, including changes in the length and number of acyl chains, changes in the number of phosphate and addition of chemical groups onto the phosphates, and changes in the number of carbohydrate moieties[29-31]. Such structural variations of lipid A can alter the ability of LPS to activate the innate immune system and elicit an immune response[32-34]. However, our results show no significant difference in IL-6 mRNA expression levels in macrophages stimulated with sonicated bacteria of either strain used, DL94 or TA131. This suggests that the difference in macrophage IL-6 response observed with live bacteria probably originates from a property unique to live bacteria rather than to LPS. Moreover, our observations indicate a possible link between the host immune response and the phylogenetic background of the bacterial strain as well as the number/type of virulence factors, as the B2 strain with the higher virulence score (TA131) elicited markedly higher macrophage IL-6 mRNA expression levels compared to those elicited by the A strain with a lower virulence score (DL94).

Our findings indicate certain differences in the macrophage IL-6 response (measured by IL-6 mRNA expression levels) elicited by two pathogenic *E. coli* strains. However, as the strains exhibit different genetic backgrounds, thorough investigation of a larger collection of strains is necessary to

ascertain correlation of characteristic strain properties and their potential to modulate innate immune responses.

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Article

Proteomics analysis of drought stress: glycoproteins in common bean

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Drought stress is one of the most important abiotic stress factors that affect plant growth and productivity. Since proteins are directly involved in plant stress response, proteomics studies with the analysis of total proteins, cellular compartments and posttranslational modifications help to unravel the relationships between protein abundance and plant stress response. Posttranslational modifications, such as glycosylation are important for changing the properties and functions of proteins and play an important role in response to stress. To analyze the importance of glycosylation during drought stress in common bean, protein extracts from stems of drought stressed and control plants of common bean were prepared and glycoproteins were enriched by lectin affinity chromatography. Glycoproteins were further separated using SDS-PAGE and the gel lanes of control and stressed plants were divided into eight slices. Proteins were digested with trypsin and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Glycoproteins with a predicted N-terminal endoplasmic reticulum targeting signal peptide and N-glycosylation consensus sequences (N-X-S/T) were determined for each gel slice. Mass spectra were manually inspected to identify glycoproteins and to determine the structure of glycans. Many MS/MS spectra for glycoproteins with marker ions of typical glycans were observed, but very small number of glycopeptides was assigned to the spectra, due to the spectral complexity. Structures of high mannose, complex and hybrid types of N-glycans were found from the spectra. Due to the complexity of spectra, quantification of glycopeptides and the determination of differences in glycan structures between plants in drought and control plants were not determined. The quantification of glycoproteins from plants in stress and controls with specific software is the aim of our further study.

INTRODUCTION

Common bean is an important crop due to the high content of proteins, dietary fibres and essential amino acids[1]. Among all the major food legumes, common bean is the world's third most important bean after soybean (*Glycine max* L.) and peanut (*Arachis hypogeal* L.)[2]. However, common bean is subjected to drought stress that reduces its yield. It is estimated that sixty percent of the world's common bean grows under rainfed conditions, and drought causes yield losses up to 80 % in some regions[3,4,5].Therefore, the development of cultivars with improved tolerance to drought is an important goal of many bean breeding programs[2]. In order to help in developing common bean cultivars exhibiting an increased drought tolerance, the improved knowledge of stress-related genes and proteins provides a necessary information in understanding the molecular basis of drought response in common bean.

Several genes and gene-like sequences whose expression responds to drought have been identified in common bean[6,7,8,9]. Unlike gene expression analyses, proteomic studies of drought response in common bean are very limited. Sengupta et al. (2011)[10] reported a systematic root proteomics approach to identify and analyze the expression patterns of differentially expressed

major root proteins of *Vigna radiata* during water deficit treatment. Drought stress-induced changes among certain structural and functional root proteins involved in reactive oxygen species detoxification, primary and secondary metabolite biosynthetic pathways and proteins associated with cell signaling. In the later report, Sengupta and Reddy (2011)[11] depicted a closer analysis of the expression patterns of these identified proteins. A schematic network of possible inter-relationships between the identified proteins and subsequent physiological effects in plant cell during medium and high water deficit regimes was shown. The study of differential proteomic analysis of drought stress response in leaves of common bean revealed proteins involved in energy metabolism, photosynthesis, ATP conversion, protein synthesis and proteolysis, stress and defence related proteins[12].

An important part of the proteomics is also the analysis of various posttranslational modifications, such is glycoslyation. Glycoproteins can function as structural elements, as signal molecules, as recognition markers for the assembly of protein complexes and can affect protein folding, solubility and stability. In plants, the main target of stress is the endoplasmic reticulum, where the proteasome-degradation and the detoxification systems are involved in stress responses[13]. Glycosylation has been shown to act on unfolded proteins and is involved in the quality control of glycoprotein assembly in the endoplasmic reticulum. Prior report suggests the importance of glycosylation for the optimal functioning of proteins involved in the plant stress response[14].

In the current study the identification of common bean stem proteins influenced by drought was performed by a proteomic approach, based on a combination of lectin affinity chromatography, one-dimensional SDS-PAGE and liquid chromatography-tandem mass spectrometry (LC-MS/MS). Our study was focused on the analysis of N-glycoslylation, because O-glycoslyation in plants is less studied that N-glycosylation. This provided the identification of some glycoproteins from spectra.

METHODS

Plant growth and stress treatments

Plants of common bean (*P. vulgaris*, cv. Tiber) were cultivated in a greenhouse under natural light, temperature and moisture conditions, as described previously[12]. Briefly, seeds were sown in pots containing a mixture of fertilized substrate (Klasmann, Germany) and vemiculite (1:1, v/v). Plants were regularly irrigated with tap water. Drought treatment was imposed on 5-week-old plants. Half of the plants were not watered whereas the second half was regularly irrigated. Plants for proteome analysis were harvested on day 17 after the beginning of water withdrawal. Both stressed and control plants were collected at each harvesting step, in order to ensure the same developmental stage for comparative proteomic analysis.

Preparation of protein extracts and gel electrophoresis

Stems with three internodes from first leaves forward were harvested, immediately frozen in liquid nitrogen and stored at -80°C until further analysis. Four replicates of stressed plants were combined together in one sample and control samples were combined and prepared in the same way. Stems were ground to a fine powder in liquid nitrogen using a pestle and mortar. Samples were transferred into a centrifuge tube and suspended in the extraction buffer containing 1x PBS (pH 7,4), 2 % SDS and complete protease inhibitors (Roche). Lysates were prepared by homogenization, sonication, centrifugation and the supernatants were collected.

To enrich the glycoproteins the stressed and control protein extracts were subjected to lectin affinity chromatography using Qproteome total glycoprotein kit (Qiagen) according to the

manufacturer's protocol. The total glycoprotein spin columns in this kit contain ConA and WGA lectins. They are used for a general enrichment of the total glycoprotein population from a sample.

Protein samples of equal concentration from stressed and control plants were separated by PAGE with pre-cast 4-12% Criterion XT gel (BioRad) in MOPS buffer (BioRad), using reagents and protocols supplied by the manufacturer (BioRad). After electrophoretic separation the gel was stained with Coomassie Brilliant Blue R-250 staining solution. The vertical line of stressed and control sample on the gel was cut into 8 slices and each gel piece was sliced into small pieces for trypsin digestion according to Shevchenko et al. (2007)[15]. Extracted peptides were analyzed immediately by MS or frozen at −80 °C.

MS and data analysis

LC-MS/MS analysis of tryptic digested peptides was performed with an Agilent 1200 nanoflow HPLC system consisting of binary pumps coupled to an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a nanoelectrospray ion source as described previously[12].

Raw data files produced in Xcalibur software (Thermo Fisher Scientific) were converted to *.mgf files
using BioWorks 3.2 software. These files were submitted to MASCOT searches within the Matrix Science web server (www.matrixscience.com). Searches were performed using the NCBInr database with a taxonomy parameter set to green plants. For the database search, parameters such as one missed cleavage site by trypsin, an MS tolerance of 7 ppm and an MS/MS tolerance of 0.05 Da, peptide charge of 2+, 3+ and 4+, oxidation of methionine as variable modification were used. Expected cut off value was set to 0.05 for removing low scoring matches. For each gel slice of control and stressed plants a protein database was established.

All identified proteins were screened for the predicted presence of an N-terminal endoplasmic reticulum targeting signal peptide, using the Signal P 4.1 program[16]. Proteins were also analyzed for the presence of predicted N-glycosylation sites using the NetGlyc (http://www.cbs.dtu.dk/services/NetNGlyc/) and Target P[17] was used to determine the predicted subcellular locations of the identified proteins.

To identify glycopeptides containing the characteristic oxonium ion of N-acetylhexosamine at m/z 204.08 spectra were manually checked. To assign detected peptide backbone masses to corresponding peptide pieces, a software program called Peptide Mass Tool[18] was used. Peptides cleaved by unspecific proteases were identified by this peptide mass tool, programmed with Perl. Specific protein database from each gel slice was used for search. This allowed us to determine predicted glycan composition and structural information, predicted peptide sequence and the glycosylation sites.

RESULTS

To analyze the stem glycoproteome of common bean under drought, the glycoproteins were enriched by lectin affinity chromatography and separated by one-dimensional SDS-PAGE (figure 1). The gel lanes of control and stressed plants were divided into 8 slices, proteins were digested with trypsin and analyzed by LC-MS/MS.

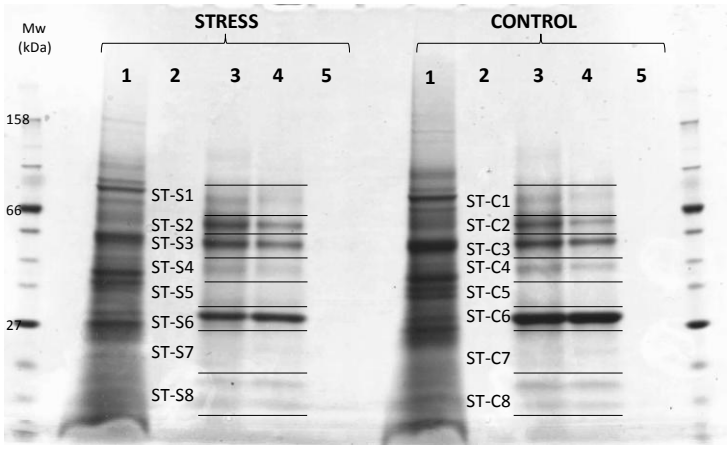

Figure 1: SDS-PAGE separation of glycoproteins from lectin cromatography for cultivar Tiber
Eight protein slices of drought stress samples (from ST-S1 to ST-S8) and eight slices of control samples (from ST-C1 to ST-C8) were excised from the gel.
1- sample binding on column; 2- washing after sample binding; 3- ME-elution; 4- SE-elution; 5- washing after elution.

Proteins were identified with the help of MASCOT search engine, where protein hits were limited with expected cut off value of 0.05. Only proteins with a predicted N-terminal endoplasmic reticulum targeting signal peptide were considered further in this study since a signal peptide is required for the co-translational translocation of nascent proteins into the endoplasmic reticulum prior to N-glycosylation[19]. Presence of the predicted N-glycosylation consensus sequences was also required for proteins in the database (table 1). There was good correlation between the number of proteins with signal peptide and the number of predicted secretory proteins, since secretory proteins are often glycosylated.

Gel no.	Number of identified proteins	Number of proteins with SP	Number of proteins in subcellular location				Number of proteins without N-X-S/T
			Secretion	Mitochondrion	Chloroplast	Other	
S1	40	25	25	0	1	13	1
C1							
S2	41	31	33	4	0	4	0
C2							
S3	37	27	30	2	0	5	1
C3							
S4	33	21	22	1	0	10	3
C4							
S5	31	16	21	1	2	7	4
C5							
S6	22	8	10	0	0	12	3
C6							
S7	18	7	8	1	0	9	3
C7							
S8	9	3	4	0	0	5	2
C8							

Table 1: The number of identified glycoproteins in stem for cultivar Tiber
The number of gels are referring to figure 1.

In order to identify glycoproteins and to determine the structure of glycans, spectrum from each protein slice was manually inspected for a specific oxonium ion at m/z 204.08. To determine the glycosylation site and glycan composition, the detection of a distinct y_1 ion was used, which is often

the most abundant y type product ions observed in the glycopeptides fragmentation[19]. Based on the charge of the y_1 ion, the charge of the glycopeptide was determined. Calculated peptide masses were used for the search against the protein database for each protein slice using the program Peptide Mass Tool. The presence of the consensus sequence for N-glycosylation (N-X-S/T; X can be any amino acid, except proline) and the correspondence with the trypsin cleavage site were requested for the selection of glycopeptides sequences. Based on the mass and charge of the peptide, the mass of glycan structure was calculated. Many MS/MS spectra for glycopeptides containing marker ions of typical glycans such as m/z 204.08, 366.13 and 528.49 were found. Despite this, very few spectra could be assigned to a peptide sequence and so the number of manually identified N-glycopeptides was relatively small. Structures of high mannose, complex and hybrid types of N-glycans were found from the spectra. Manual comparison of glycopeptides and glycan structures from spectra was difficult due to the technical challenges associated with the analysis at this approach.

DISCUSSION

With this study lectin affinity chromatography was successfully applied to enrich glycoproteins from total protein extracts of control and stressed plants of common bean under drought. The enriched glycoproteins were separated by SDS-PAGE and excised slices were analyzed by LC-MS/MS. Many MS/MS spectra for glycoproteins with marker ions of typical glycans were observed, but very small number of glycopeptides was assigned to the spectra. This reflects the labile nature of the glycan-peptide linkage and the relative fragile internal glycan bonds during fragmentation compared to peptide bonds, which often results in little or uncertain information concerning peptide sequence, glycosylation site and glycan structure[19]. Low ionization efficiency, large mass, structural complexity and low abundance of glycopeptides make the reading from spectra even worse. Glycoproteins with low abundance are difficult to detect in complex mixtures, due to the large dynamic range of glycoproteins in biological samples. The reason for difficult and uncertain comparison of glycan structures and glycopeptides from spectra for stressed in control samples lies in the complex interpretation and slightly bad quality of spectra in our study. In spite of all that, manual inspection of spectra from each protein slice allowed the detection of proteins with different glycan structures. For example, peptide with mass 1193.59 was identified as moncopper oxidase with high mannose type of glycans with different number of mannose units in sample ST-S1, ST-C1. In addition, the same protein could present a variety of glycan structures and the same glycan structures are linked to different proteins. Overall, structures of high mannose, complex and hybrid types of N-glycans were found by manual inspection from spectra. Due to the complexity of spectra, the information about quantification of glycopeptides from stressed and control plants are difficult to determine. For the same reason, differences in glycan structures between plants in drought and control plants could not be presented in this study.

The aim of our further study is the quantification of glycoproteins from plants in stress and controls with specific software. The identification of those proteins that exhibited changed abundance under drought stress will be exposed.

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Abstract

Development of genetic map of common bean (Phaseolus vulgaris l.) for quantitative trait loci detection involved in response to drought

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Because of the climate changes, higher temperatures and lower rainfall, drought every year affects more than half of the common bean production worldwide[1]. The development of varieties with a strong tolerance to abiotic stress is the primary goal of many breeding programs of common beans in the world[2, 3]. Common bean is susceptible to drought, but the mechanisms of its response are still not well characterized.

The population of 80 recombinant inbred lines (RILs) of F8 generation based on the crossing of drought resistance cultivar Tiber with the susceptible cultivar Starozagorski čern was grown in a greenhouse under controlled conditions. At the stage of flowering RILs were stressed by withholding irrigation. Morphological and physiological parameters that discern the two parental lines, e.g. grain and flower color, days to flowering, seed yield, weight of 100 seeds, water potential and photosynthetic fluorescence, were scored at the different stage of drought. Samples of each RIL were examined to identify polymorphisms for every SSR and AFLP marker that distinguished the two parental lines. DNA fragments were amplified by PCR, and visualized on an agarose gel and capillary electrophoresis. Overall 345 microsatellites (25% polymorphic) and 240 AFLP markers (10% polymorphic) were tested. Based on polymorphisms 69 microsatellites were placed to genetic map of common bean.

QTL analysis will be employed to identify informative markers that are closely linked to loci involved in the response of common bean to drought stress. These markers will significantly improve our breeding programs aimed at development of drought tolerant bean varieties.

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ABSTRACTS of POSTERS

GENE–ENVIRONMENT INTERACTION

Klara Hercog, Metka Filipič, Bojana Žegura

Genotoxic potential of the binary mixture of cyanotoxins microcistin-lr and cylindrospermopsin

Daša Medveščeк, Katja Zeme, Katja Molan, Darja Žgur-Bertok, Irena Zdovc, Marija Trkov, Jerneja Ambrožič Avguštin

Molecular comparison of *Escherichia coli* isolates from human respiratory tract and strains isolated from poultry in Slovenia

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Proteomic analysis and physiological response of hop (*Humulus lupulus* l.) to drought stress

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Abstract

Genotoxic potential of the binary mixture of cyanotoxins microcistin-lr and cylindrospermopsin

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Increased eutrophication of water bodies promotes the blooming of cyanobacteria that are hazardous due to the production of cyanotoxins. Microcistin-LR (MCLR) is one of the most widespread cyanotoxins classified as possible human carcinogen, while cyindrospermopsin (CYN) has only recently been recognized as health concern. Both cyanotoxins are genotoxic, but the mechanisms of their action are different. MCLR induces DNA damage via reactive oxygen species formation, while CYN is pro-genotoxic and probably directly interacts with DNA (1). They are ubiquitously present in the water environment and can often be found together in surface freshwaters. As it is known that complex mixtures can evoke more pronounced adverse effects than individual compounds, we studied the genotoxic potential of the binary mixture of the two cyanotoxins.

Human hepatoma cells (HepG2) were exposed to non-cytotoxic graded doses of CYN (0,01-0,5 µg/ml), single dose of MCLR (1µg/ml) and their combinations for 24 hours. No significant increase in DNA damage in cells treated with pure cyanotoxins or their binary mixtures was detected with the comet assay and γH2AX foci assay. A significant increase of micronuclei formation was observed at the highest dose of CYN alone and combined with MCLR, while MCLR alone had no significant effect suggesting that MCLR does not affect genotoxic activity of CYN. It can be concluded that at non-cytotoxic concentrations the binary mixtures of CYN and MCLR does not exhibit synergistic or potentiating genotoxic effects. Our future plan is to study the combined effects of MCLR and CYN at the transcriptional level.

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Abstract

Molecular comparison of *Escherichia coli* isolates from human respiratory tract and strains isolated from poultry in Slovenia

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Escherichia coli strains can cause intestinal and extraintestinal infections in humans. Extraintestinal pathogenic *E. coli* (ExPEC) are frequently implicated in urinary tract infections, septicemia and also in colibacillosis in poultry - in such case they are labeled avian pathogenic *E. coli* (APEC). Recently it has been suggested that food represents an important source of *E. coli* strains, which can enter the body of animals and humans and cause various intestinal and extraintestinal infections. In our study we analyzed 162 *E. coli* strains isolated from poultry and compared them with 284 strains, isolated from human respiratory tract samples. The phylogenetic groups and presence of virulence genes *fimH, ompT, fluA*, *vat*, *kpsMT*II, *usp*, *iha* and *sat* were detected by PCR. Further, all isolates were screened for the presence of β-lactamases from group CTX-M, TEM and SHV to estimate the resistance potential of the analyzed isolates. Among the 162 *E. coli* poultry isolates 55 (34%), 51 (31,5%), 19 (12%), 19 (12%), 17 (10,5%) and 2 (1%) segregated to phylogenetic groups B1, D_2, A_0, D_1, A_1 and $B2_3$, respectively. In comparison none of the human respiratory tract (HRT) isolates belonged to phylogenetic group B1, whereas, 46 (16%) isolates were placed into group D_2, 5 (1,5%) into group A_0, 18 (6,5%) into D_1, 38 (13,5%) into A_1 and 166 (58,5%) isolates into phylogenetic group $B2_3$. Gene *fimH* was detected in 153 (59%) poultry isolates, *ompT* in 124 (43%), *fluA* in 47 (12%), *vat* in 38 (12%), *kpsMT*II in 24 (14%), *usp* 10 (6%) and *iha* in 29 (18%) isolates, whereas gene *sat* was not found. In comparison, *fimH*, *ompT*, *fluA*, *vat*, *kpsMT*II, *usp*, *iha* and *sat* genes were present in 92,5%, 56,5%, 80,5%, 2%, 65,5%, 66%, 62% and 64% of the human respiratory tract isolates, respectively. Additionally 95 (59%), 40 (25%) and 44 (27%) poultry isolates were positive for genes $bla_{CTX\text{-}M}$, bla_{TEM} and bla_{SHV}, and additionally genes for CTX-M, TEM, and SHV enzymes were found in 91%, 56% and 9% HRT isolates, respectively. We conclude that the percentage of isolates belonging to different phylogenetic groups and virulence gene content differ clearly in poultry and HRT isolates.

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Abstract

Proteomic analysis and physiological response of hop (*Humulus lupulus* l.) to drought stress

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Drought is one of the most important constraints on the growth and productivity of many crops, including hops (*Humulus lupulus* L.). To improve our understanding of the complex mechanisms involved in the response of hop to drought stress, a proteomic approach was used to identify proteins in the leaves of two cultivars (Aurora and Savinjski golding) differing in their response to drought. A growth experiment was carried out in 2013 and 2014. Measurements of physiological parameters (gas exchange techniques, fluorescence, water potential measurements) gave a better insight into the drought response of plants and enabled the selection of plants for proteomic analysis. Proteins in leaves were extracted from four biological replicates, the concentration of proteins was measured with a 2D Quant kit, while 2D-DIGE technology was used to compare differences in protein abundance between control and stressed plants. Physiological measurements and preliminary proteomic results of the experiment will be presented.

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ABSTRACTS of POSTERS

MOLECULAR BASIS OF DISEASES

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Proteolytic cascades in glioblastoma and mesenhymal stem cells direct cross-talk

Alja Gabor, Katja Repnik, Uroš Potočnik, Matej Horvat-Šprah

Genetic risk factors for recurrence of primary colorectal cancer

Urška Gabor, Katja Repnik, Uroš Potočnik

Association of polymorphisms and expression of selected genes with response to treatment of Crohn's disease patients with adalimumab

Davor Obradović, Matej Butala, Nina Orehar, Rok Gašperšič and Peter Maček

Prevalence of virulence factors of Aggregatibacter actinomycetemcomitans in Slovene population

Petra Perin, Marko Bitenc, Janez Bernik, Matjaž Stanonik, Jernej Luznar, Sara Redenšek, Borut Štrukelj

Non-invasive prenatal testing for Down syndrome by analysing specific methylated regions of foetal DNA

Žiga Strmšek, Tanja Kunej

Catalogue of microRNA genes silenced by DNA methylation in cancer: facilitating development of novel biomarkers

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Abstract

Proteolytic cascades in glioblastoma and mesenhymal stem cells direct cross-talk

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Glioblastoma multiforme (GBM) is the most invasive brain tumour, characterised by single cell invasion into normal brain parenchyma reflected in GBM recurrence and poor patients' survival. This process is assisted by tumour microenvironment, where the infiltrating mesenchymal stem cells (MSCs) play a role, which is not yet well understood. Demonstrating intrinsic tumour-homing ability (1), MSCs can be recruited to the tumour from endogenous tissue niches or from bone marrow (BM) *via* blood circulation. We recently reported that GBM invasion was enhanced in directly co-cultured MSC/GBM cells, which form a structural as well as functional syncytium (2).

Aim: In this study we wanted to elucidate the pathway of proteolytic cascade in MSC/GBM direct co-cultures. As it is known that proteases are crucial for GBM invasion (3) we investigated their expression at protein level in MSCs and GBM cells when they were co-cultured together.

Methods: We evaluated the expression of proteases and their receptors in direct co-cultures of BM-MSCs and U-87 MG cells *in vitro* by flow cytometry and immunocytochemistry.

Results and Conclusions: Among tested candidate proteases and their receptors, matrix metalloproteinases 9 and 14 (MMP9 and MMP14) were found to be up-regulated in GBM cells when they were co-cultured with BM-MSCs. Meanwhile, all candidate proteases and their receptors were down-regulated in BM-MSCs in direct co-cultures. Results show up-regulation of MMPs in directly co-cultured GBM cells, which probably cause increased GBM invasion in MSC/GBM cells complex. In contrast, MSCs have decreased expression of proteases indicating less invasive phenotype of MSCs in MSC/GBM direct co-cultures. To get deeper insight in MSCs and GBM cells direct cross-talk we are planning to perform gene expression analysis of candidate proteases genes and to analyze secreted proteases from MSC/GBM co-cultures.

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Abstract

Genetic risk factors for recurrence of primary colorectal cancer

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Colorectal cancer (CRC) is a malignant tumour disease with high incidence and mortality. Within three years after resection of the primary tumor in nearly one third of cases local recurrence occurs or leads to metastasis in distant organs. The development of the disease significantly affects hereditary SNPs in genes, associated with CRC as well as SNPs in genes that contribute to the occurrence of the distant metastases.

The aim of our study was to determine the role of selected SNPs in the genes MMP7 (rs11568818) and MACC1 (rs1990172) in the glow of the lymph nodes and CRC recurrence. SNPs were selected by following previous studies that confirmed association between these SNPs and increased risk for liver metastases development or their progress.

DNA was isolated using commercial kits (Roche and BiOstic) from FFPE tissue samples of the intestinal mucosa and genotyped using High Resolution Melting (HRM) and Polymorphism Chain Reaction followed by Restriction Fragments Length Polymorphism (PCR – RFLP) technique. We compared the genotype and allele frequencies of patients with CRC with those of healthy individuals in Slovenian population. The allele and genotype frequencies between the patients with CRC depending on the stage of the TNM classification, CRC recurrence and some other clinical parameters were also compared.

Increased frequency of GG genotype in the group of patients compared to healthy controls in the case of both SNPs was detected. For SNP rs11568818 in the case of AA genotype we found significant association with increased risk of the spread and recurrence of the disease ($p = 0.03$). For the SNP rs1990172 we found no statistical significant association.

Our study, together with many others, emphasise the importance of SNPs to predict increased risk for recurrence of CRC and allows more appropriate treatment adjustments for each individual.

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Article

Association of polymorphisms and expression of selected genes with response to treatment of crohn's disease patients with adalimumab

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Crohn's disease (CD) is an autoimmune disorder of unknown etiology. Currently, the greatest potential for successful management of the disease is exhibited by biological therapeutics, including adalimumab (ADA). Pharmacogenetic studies concerning ADA, however, are still in an early stage. The aim of our study was to determine whether there was a correlation between response to treatment with ADA and selected single nucleotide polymorphisms (SNPs) as well as expression of selected genes in Slovenian CD patients. Selected SNPs and genes have shown a strong association with CD in previous studies, are involved in drug metabolism, associated with immune response and inflammation or with related complex diseases. Genotypes for selected SNPs were obtained from "Immunochip" microarray. Gene expression was measured at weeks 0, 4, 12, 20 and 30 after initiation of treatment using real-time polymerase chain reaction. Comparison of patients' demographic data showed a significantly lower percentage of smokers amongst patients with good response compared to patients with poor response (p=0.042). Association analysis for rs10919563 and rs2241880 showed that good responders have significantly different frequency of G/G genotype (87.5% and 25.0%) in week 30 (p=0.045 for rs10919563 and p=0.025 for rs2241880) compared to poor responders (65.0% and 55.0%). We have determined a difference in expression of *SLC22A4*, *PSMD3*, *AHSA2*, *RPRD2* and *PUS10* between good and poor responders prior to treatment. In three genes (*ATG16L1*, *RPRD2* and *PUS10*) expression was significantly changed relatively to week 0 throughout the treatment. The most statistically significant results were obtained for *ATG16L1*, with a p-value ranging from 1.0×10^{-6} to 2.2×10^{-18} (week 12). When comparing gene expression between good and poor responders according to ΔIBDQ values, we found statistically significant differences between the groups for *ATG16L1* and *SLC22A5* in week 12. This study identified new pharmacogenomic biomarkers, which could be helpful in establishing a more tailor-made approach for CD treatment with fewer side effects.

INTRODUCTION

Crohn's disease (CD) is a relapsing inflammatory condition, mainly affecting the gastrointestinal tract. Genome wide association studies identified a large number of susceptibility loci that, when triggered by environmental factors, result in a disturbed innate and adaptive immune response towards a diminished diversity of commensal microbiota [1,2]. The disease is not yet curable. However, it can be managed by a combination of therapeutics, dietary changes or surgery. Studies show that CD patients have elevated levels of tumor necrosis factor alpha (TNF-α), a cell signaling molecule responsible for chronic relapses. Use of TNF-α inhibitors reduces the amount of TNF-α that can bind to its receptors. Blocking the formation of the ligand-receptor complex inhibits intracellular signaling cascades, which produce inflammatory proteins. The main side effect of anti-TNF therapeutics is a major risk of infection and, in rare cases, development of other autoimmune diseases [3]. Currently, the newest and most promising biological drug on the Slovenian market is Humira® with adalimumab (ADA) as the active ingredient. ADA is a human monoclonal antibody against TNF-α [4]. Several clinical trials tested efficacy and safety of ADA, including CHARM [5], CLASSIC-I [6], CLASSIC-II [7] and EXTEND [8] trials. In addition to clinical trials, pharmacogenetic studies are of paramount importance. To date several pharmacogenetic studies for ADA have been conducted concerning rheumatoid arthritis [9] and psoriasis [10, 11]. Concerning CD, however, there have only been

pharmacogenetic studies for infliximab (IFX). IFX is a himeric monoclonal antibody. Consequently, a significant proportion of patients tend to lose response to it or develop severe adverse effects. Since IFX and ADA share similar mechanisms of action, biomarkers associated with response to IFX should in theory apply to ADA as well. [12, 13]

The aim of our study was to establish whether there was a correlation between treatment with ADA and selected single nucleotide polymorphisms (SNPs) or expression of selected genes in Slovenian CD patients.

METHODS

The study included 107 patients with a reliable clinically and histopathologically confirmed diagnosis of CD, who were treated with ADA. Inclusion criteria were:

- Lack of response to standard therapy. Patients who received 5-aminosalicylates (4.5 g/day for at least 2 months); antibiotics - metronidazole (1.2 g/day) and/or ciprofloxacin (1 g/day) for at least 1 month; 6-methyl-prednisolone (corticosteroids) (42 mg/day for at least 2 months); azathioprine (2.5 mg/kg/day for at least 6 months).
- Lack of response to prior treatment with biological drug infliximab (IFX).
- Development of severe adverse effects or the occurrence of complications such as formation of fistulae.

208 healthy voluntary blood donors were also enrolled in the study. The study was approved by Republic of Slovenia National Medical Ethics Committee (NMEC).

Prior to as well as after 4, 12, 20 and 30 weeks of treatment, we collected patients' blood samples and measured C-reactive protein (CRP) values. Positive response was defined as a drop in CRP value to a normal level (c<3 mg/L) or a decrease of over 25%. Simultaneously, patients also filled out the "Inflammatory Bowel Disease Questionnaire" (IBDQ), which we used to calculate ΔIBDQ values. Values between 170 and 190 points (or an increase of minimum 22 points) were considered a state of remission. Whole blood was used for total genomic DNA isolation. First, we isolated lymphocytes using Ficoll-Paque Plus™ (GE Healthcare, Uppsala, Sweden) gradient centrifugation, according to the manufacturer's instructions. We isolated RNA and DNA using TRI reagent (Sigma, Steinheim, Germany), and dissolved it in water to a final concentration of c = 50 ng/µL. We used the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) for conversion of RNA to single stranded, complementary DNA (cDNA), which we used for gene expression measurements.

Upon literature review, we selected 12 candidate genes and 6 SNPs, located in different loci, which had previously been associated with CD and other complex immune diseases, were involved in drug metabolism and immune response mechanisms and/or had been associated with anti-TNF drug response.

Selected SNPs had been genotyped using "Immunochip" (iChIP) platform. [14] Using bioinformatic tools (Universal ProbeLibrary Assay Design Center (Roche Applied Biosystems), AceView, Entrez Gene, IDT Oligo Analyzer) we dimensioned primers and selected the appropriate isoforms for gene expression measurements. We performed qPCR reactions using LightCycler 480 (Roche) for cDNA analysis. Reaction volumes were 10 µL, each containing 50 ng of cDNA, SybrGreen Master Mix (Fermentas) and optimized concentrations of each nucleotide primer. PCR conditions were as follows: preincubation at 95°C for 10 min, followed by 40 cycles of 15 s denaturation at 95°C, 30 s annealing at 55-65 °C, 30 s extensions at 72 °C, and a high resolution melting step with a ramp-rate 0.11 °C/s. We also measured expression of reference genes (*B2M* and *GAPDH*).

Data analysis was carried out using SPPS Statistics 20.0 and GraphPad Prism 6. Data distribution was determinated using Kolgomorov-Smirnov test for groups larger than 50 samples and Shapiro-Wilk test for smaller groups. Genotype and allele frequencies were calculated for controls and patients (altogether as well as good and poor responders (according to ΔIBDQ and ΔCRP values) separately). χ^2 and two-sided Fischer's exact test were used to calculate the significance of the difference in allele and genotype frequencies between (a) patients and controls and (b) good and poor responders. We also calculated the odds ratio (OR) for CD with 95 % confidence intervals (CI). $2^{-\Delta\Delta Ct}$ method was used for gene expression calculations. Average Ct values of whole samples were used as a calibrator. We used nonparametric Mann-Whitney test for comparison of gene expression between: (a) samples prior to and after 4, 12, 20 and 30 weeks of treatment and (b) samples of good and poor responders, according to ΔIBDQ and subjective assessment of the patient's physician. We used the Wilcoxon paired test to compare samples of the same individuals prior to and after 4, 12, 20 and 30 weeks of treatment. Results with $p<0.05$ were considered statistically significant.

RESULTS

Comparison of demographic data revealed a significantly ($p = 0.042$) lower proportion of smokers amongst good responders (31.7 %) compared to poor responders (52.9 %)

We found that SNP rs2395185, located near *HLA-DQA1*, is associated with CD. G/G genotype frequency was found to be significantly ($p = 0{,}011$) lower in patients (44.2 %) compared to controls (60.6 %). Allele frequency for G allele was also significantly ($p = 0{,}023$) lower in patients (67.5 %) compared to controls (77.6 %).

When comparing genotype and allele frequencies for rs10919563 (*PTPRC*) with response to ADA according to ΔIBDQ, we found that good responders had significantly ($p = 0{,}045$) higher G/G genotype frequencies (87.5%) in week 30 of treatment compared to poor responders (65.0 %). For rs2241880 (*ATG16L1*) we found that good responders had significantly ($p = 0{,}025$) lower G/G genotype frequencies (25.0 %) compared to poor responders (55.0 %) in week 30 of treatment. Conversely, according to ΔCRP for rs2241880 good responders had significantly ($p = 0{,}017$) lower A/A genotype frequencies (40.7 %) compared to poor responders (63.6 %) in week 12 of treatment.

Mann-Whitney test showed a statistically significant difference in gene expression relatively to week 0 in at least one of the weeks in all 12 selected candidate genes. Associations with the highest significance are presented in Table 1. Arrows indicate whether expression increased or decreased relatively to week 0. Wilcoxon paired test yielded similar, slightly less significant results.

Comparison of baseline expression levels of selected genes, i.e. prior to treatment, between good and poor responders showed differences in expression in five genes (*SLC22A5, PSMD3, AHSA2, RPRD2* and *PUS10*). Corresponding *p*-values are given in Table 2. It is clear from Figures 1-5, that expression of the aforementioned five genes is lower in patients with good response, compared to patients with poor response.

Gene	*p*-value			
	Week 4	Week 12	Week 20	Week 30
CCNY	0,089	4.1×10^{-4} ↓	0,034 ↓	0,076
ATG16L1	4.3×10^{-11} ↓	2.2×10^{-18} ↓	9.5×10^{-7} ↓	1.0×10^{-6} ↑
AHSA2	0,078	0,032 ↑	2.2×10^{-4} ↑	0,013 ↑
CPEB4	1.1×10^{-15} ↑	6.2×10^{-7} ↑	0,004 ↑	0,070
RPRD2	3.0×10^{-10} ↓	9.4×10^{-4} ↓	0,050 ↓	1.2×10^{-4} ↓
PUS10	0,004 ↑	0,017 ↑	2.2×10^{-4} ↑	0,0012 ↑

Table 1: Most significant p-values obtained using Mann-Whitney test for comparison of gene expression prior to and after 4, 12, 20 and 30 weeks of treatment.

Gene	*p*-value
SLC22A5	0,013
PSMD3	0,042
AHSA2	0,004
RPRD2	0,008
PUS10	0,030

Table 2: p-values obtained using Mann-Whitney test for comparison of gene expression between good and poor responders prior to treatment.

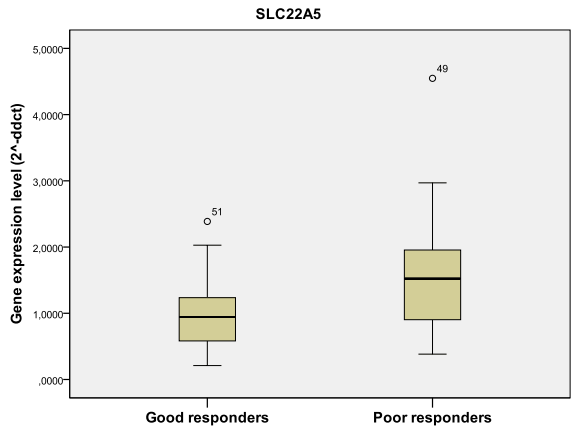

Figure 1: Expression level of SLC22A5 in good and poor responders

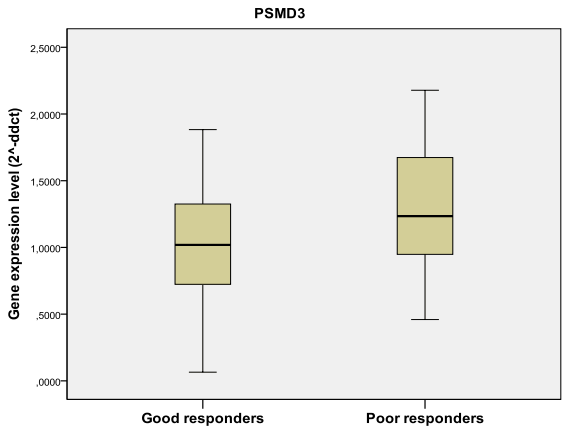

Figure 2: Expression level of PSMD3 in good and poor responders

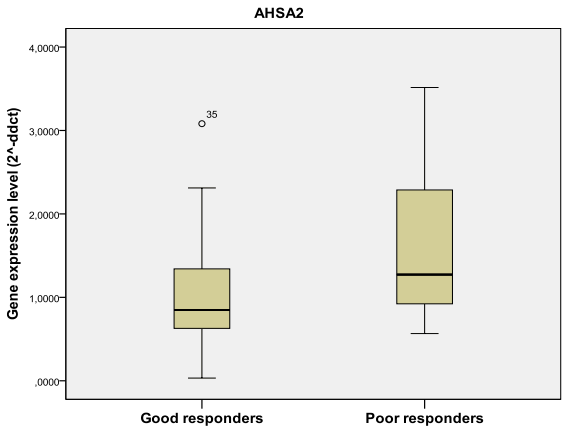

Figure 3: Expression level of AHSA2 in good and poor responders

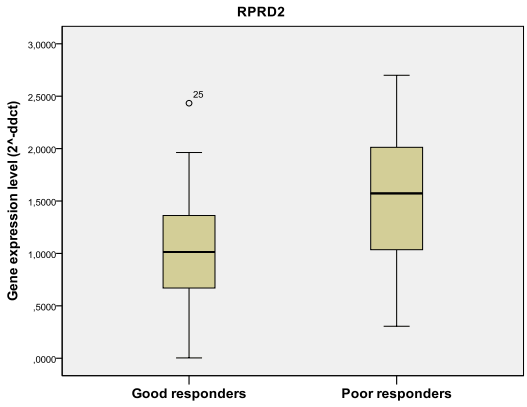

Figure 4: Expression level of RPRD2 in good and poor responders

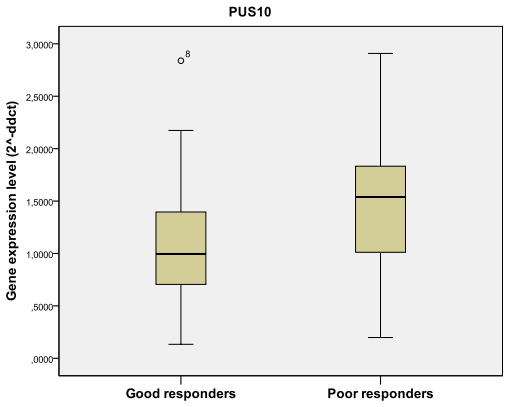

Figure 5: Expression level of PUS10 in good and poor responders

Comparison of gene expression levels in weeks 4, 12, 20 and 30 of treatment between good and poor responders according to ΔIBDQ revealed two statistically significant associations in week 12. Expression levels of ATG16L1 and SLC22A5 were significantly higher in good responders compared to poor responders, with *p*-values of $p = 0.016$ and $p = 0.042$, respectively.

DISCUSSION

This study analyzed association of demographic data, selected SNPs and gene expression with response to treatment with ADA. We report a replication of correlation between smoking and response to therapy in CD patients as well as novel correlations between SNPs and gene expression levels with response to treatment with ADA.

We have determined that smokers are less likely to produce a positive response to treatment with ADA. This is in concordance with results of previous studies, which have shown that smokers have higher susceptibility for development of CD, relapse and poor response to treatment. [13, 15]

We have also replicated a previous association [16] of rs2395185 (*HLA-DQA1*) with CD. The protein encoded by *ATG16L1* gene is a part of a large protein complex, which is necessary for autophagy, the major process for targeted degradation of intracellular components. Defects in this

gene are known to cause susceptibility to CD. [17, 18] G allele had been found to be the risk allele for the disease. Our study confirmed that poor responders have higher G/G genotype frequencies compared to good responders.

Gene expression measurement results only showing a statistically significant correlation in one or two of the weeks, suggest only a short or long-term response. Analysis of three of the genes (*ATG16L1*, *RPRD2* and *PUS10*) showed both short and long-term response. In *ATG16L1*, expression was significantly reduced in weeks 4, 12 and 20 compared to week 0 and elevated in week 30. Reduction in the first 20 weeks could be interpreted as an adjustment period to the therapy.

The five genes that exhibited lower expression levels in good responders compared to poor responders prior to treatment, could potentially be used as predictors of response to ADA.

Our results represent additional genetic evidence concerning the involvement of selected SNPs and genes in the disease pathogenesis and/or adalimumab's mechanism of action. These pharmacogenomic markers could potentially be used as predictors of response and therefore lead to a more appropriate choice of therapy.

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Abstract

Prevalence of virulence factors of Aggregatibacter actinomycetemcomitans in Slovene population

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Aggregatibacter actinomycetemcomitans (AAC) is a small, fastidious, non-motile, non-encapsulated, slow-growing, capnophilic, Gram-negative cocco-bacillus. It´s habitat is human mouth and can be recovered on culture of oral secretions in up to 20% of healthy people and in the great majority of those with localized juvenile periodontitis (LAP). The most studied toxins of AAC are leukotoxin (LtxA) and cytolethal distending toxin (CDT), they both play a role in immune evasion, but probably differ in their target-cell specificity and pattern of expression during disease.

The aim of the study is to assess the prevalence of mentioned virulence factors of *Aggregatibacter actinomycetemcomitans* isolated from subgingival pocket from 15 patients diagnosed with LAP. By comparing the results of the survey with other published clinical data from abroad, we might better understand the disease and hopefully obtain some new genetic and biochemical insight.

Localized juvenile periodontitis (LAP) is an oral disease caused by *Aggregatibacter actinomycetemcomitans*. By genetically analysing the virulence factors of the strains obtained from patients with LAP we hope to obtain better understanding of the disease.

Main method for identifying the presence of virulence factors in strains is isolating bacterial DNA and specifically multiplying sequences in the bacterial genome by PCR (polymerase chain reactions).

Strains of *Aggregatibacter actinomycetemcomitans* isolated from patients diagnosed with LAP contain almost all virulence factors.

By knowing which virulence factors are present in a strain of *Aggregatibacter actinomycetemcomitans* obtained from potential patient we can better formulate the treatment.

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Article

Non-invasive prenatal testing for Down syndrome by analysing specific methylated regions of foetal DNA

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Down syndrome is a chromosomal abnormality which occurs as a result of additional copy of chromosome 21 (T21). Currently, amniocentesis still represents a gold standard for prenatal diagnosis of chromosomal abnormalities. The method is invasive, unpleasant and leads to a miscarriage in approximately 1 % of the cases. In contrary, non-invasive prenatal techniques analyse foetal DNA from maternal blood and represent highly accurate screening methods for common trisomy including chromosome 21. The methylation patterns are changing during the development and there are DNA regions which are differently methylated in foetus compared to the pregnant women. The aim of our study was to test a highly sensitive and highly specific method for the detection of chromosomal trisomy 21 by analysing specific regions of DNA, which are differently methylated in foetus compared to maternal DNA. In our study, 20 blood samples form women in 12th to 18th week of pregnancy with known diagnosis for chromosomal trisomy were included. First of all we have tested the stability of cell free DNA in the blood sample in different tubes. Bloods samples were centrifuged to remove cells and DNA (foetal and maternal) was isolated from plasma. Further, DNA was fragmented by sonication and immunoprecipitation of methylated parts of DNA was performed. DMR regions of chromosome 21 from the whole and from imunoprecipitated DNA samples were amplified and quantified using RT-PCR. The statistic algorithm was developed to determinate foetal fraction of DNA and D-score calculation. To distinguish between foetus with trisomy and diploid foetus the optimal cut-off D-score value was determinate. Cell free DNA of all pregnancies contained at least 6 % foetal fraction. D-score 10.7 was found as optimal cut off value which showed 100 % accuracy of determination Down syndrome. Analysis of DMR regions of foetal DNA from maternal blood represents fast, non-invasive, highly accurate and cost-effective approach for detecting trisomy 21 and can be performed already in the twelfth week of pregnancy and is therefore a promising screening method for Down syndrome and other chromosomal abnormalities and might be used in future in routine clinical practice.

INTRODUCTION

Aneuploidy is a condition in which chromosomal number differs from wild type by part of chromosomal set. Aneuploidy originates during cell division when the chromosomes do not separate properly between two cells. Most aneuploidies arise from errors in meiosis, especially in maternal meiosis I. Because each chromosome contains hundreds of genes, the addition or loss of a single chromosome disrupts the existing equilibrium in cells and in most cases is not compatible with life[1]. In humans, the most common aneuploidies are trisomies, which represent about 0.3 % of all life births[2]. Down's syndrome, caused by trisomy of chromosome 21, is considered to be the most frequent etiology of mental retardation, with an incidence of 1 in 700 child births in all populations worldwide. Talking for Slovenia, each year about 20 children are born with Down syndrome. Patients with this trisomy are characterised by typical facial features, mental retardation and are susceptible to various diseases[3,4].

At the moment, few different non-invasive screening prenatal tests are available. The most important are: biochemical marker screening of maternal blood and foetal nuchal translucency. Conventional screening tests could be performed between 11th and 13th week of pregnancy, however, the detection rate is below 80 % and in addition, there are a lot of false positive results.

Further, the high risk pregnancies are redacted to the diagnostic investigations. Diagnosis is currently performed by one of three possible methods: amniocentesis, chorionic villus sampling (CVS) or cordocentesis. However, mentioned procedures are invasive and associated with a considerable risk of foetal loss (approximately 1%). In some cases, it is necessarily to repeat the procedure, which just doubles the risk of foetal loss. Expect CVS, they are also performed in the later weeks of pregnancy, which means that abortion before 18th week of pregnancy is not possible.

In contrary, non-invasive prenatal techniques analyse foetal DNA from maternal blood and represent highly accurate screening methods for common trisomy including chromosome 21, 18, 13 and aneuplodies of sex chromosomes[5]. Possibilities of non-invasive prenatal testing for detection of genetic abnormalities were indicated in year 1997, when the presence of foetal DNA in maternal blood was discovered[6]. The main question was, how to distinguee between maternal and foetal DNA in purpose to detect foetal chromosomal abnormalities. Progress of the technology, especially whole genome sequencing, enables sequencing of whole cell free DNA present in the sample of maternal blood and the detection of chromosomal abnormalities without distinguee between maternal and foetal DNA. The techniques base on determination the chromosome of origin for each sequenced fragment in the sample, counting fragments which belong to each chromosome and calculation the proportion of fragments in sample which originate from chromosomes of interest. If the proportion of curtain chromosome exceeds expected proportion, it is considered that there is a trisomy of that chromosome in foetus[7]. Current NIPT methods are very sensitive and specific with detection rate above 99.5 % for listed chromosomal abnormalities and are completely safe for the pregnant woman and foetus[5,7].

Our genes are “activated” or “inactivated” by chemical process: methylation of cytosine on specific DNA regions. Because the methylation patterns are changing during the development, there are DNA regions which are differently methylated (DMR) in foetus compared to the pregnant women. Therefore, whole DNA from maternal blood sample can be isolated and then foetal and maternal DNA can be separated (based on DMRs) with procedure, called methylated DNA immunoprecipitation (MeDIP), which permits highly efficient enrichment of methylated DNA. An antibody specific for methylated cytosine could be used to immunocapture methylated genomic fragments and in the final stage real-time PCR is used for DNA quantification In case of Down’s syndrome, there is higher percentage of foetal DNA from 21 chromosomes[4]. The test is supposed to be almost if not totally 100 percent reliable and is in addition very cost effective.

The aim of our study was to test a highly sensitive and highly specific method for the detection of chromosomal trisomy 21 by analysing specific regions of DNA, which are differently methylated in foetus compared to maternal DNA.

METHODS

In our study, 20 blood samples form women in 12th to 18th week of pregnancy with known diagnosis for chromosomal trisomy were included. Bloods samples were centrifuged to remove cells and cell free DNA (foetal and maternal) was isolated from plasma. DNA was isolated with phenol/chloroform/isoamyl alcohol extraction according to XL GenDNA Extraction Module Kit protocol. Further, fragmentation was performed using the Bioruptor at “low” power using the following cycles: 15 seconds “on” and 15 seconds “off” for a total time of 10 min. The methylated

fragments were separated from non-methylated fragments in the sample using MeDIP kit (Diagenode) according to the kit protocol.

Table 1: Primers used for rt-PCR reaction

ID	Sequence	Concentration required
oligoJW102	GCGGTGACCCGGGAGATCTGAATTC	50 uM
oligoJW103	GAATTCAGATC	50 uM
EP1L	CCAGGCAAGATGGCTTATGT	300 nM
EP1D	ACCATGCTCAGCCAATTTTT	300 nM
EP4L	CTGTTGCATGAGAGCAGAGG	900 nM
EP4D	CGTCCCCCTCGCTACTATCT	900 nM
EP5L	TGCAGGATATTTGGCAAGGT	450 nM
EP5D	CTGTGCCGGTAGAAATGGTT	450 nM
EP6L	TGAATCAGTTCACCGACAGC	900 nM
EP6D	GAAACAACCTGGCCATTCTC	900 nM
EP7L	CCGTTATATGGATGCCTTGG	750 nM
EP7D	AAACTGTTGGGCTGAACTGC	750 nM
EP10L	CCACATCCTGGCCATCTACT	300 nM
EP10D	TTCCACAGACAGCAGAGACG	300 nM
EP12L	ATTCTCCACAGGGCAATGAG	300 nM
EP12D	TTATGTGGCCTTTCCTCCTG	300 nM
HYP1L	CAGGAAAGTGAAGGGAGCTG	300 nM
HYP1D	CAAAACCCAATGGTCAATCC	300 nM
U1L	AAGGTGCCCAATTCAAGGTA	300 nM
U1D	CTTCCCCACCAGTCTTGAAA	300 nM

After literature review, DMRs of chromosome 21 were selected. Selected regions from the whole and from imunoprecipitated DNA samples were amplified and quantified using RT-PCR.

The quantity of selected DMRs was calculated. Using rt-PCR we obtained Ct – values (threshold cycle) for each sample for both – whole sample input & immunoprecipitated sample. Further, the ct values normalised. For each DMR we calculated ratio between: Norm ΔC_T $^{\text{Sample}}$ $^{\text{(Normal or T21)}}$ and Median (Norm ΔC_T $^{\text{Normal}}$). The value of ratio for each DMR we used for development of algorithm for D-value calculation with discriminant analysis.

RESULTS

Table 2: D-score of training set data

SAMPLE	Diagnosis	D-vrednost
1	Normal	-3,57
2	Normal	0,13
3	Normal	4,82
4	Normal	2,63
5	Normal	0,94
6	Normal	0,96
7	Normal	3,54
8	Normal	-1,67
9	Normal	0,59
10	Normal	-2,53
11	Normal	-2,99
12	Normal	-2,85
13	Normal	-2,26
14	Normal	2,94
15	Normal	-0,58
16	Normal	-1,17
17	Normal	6,19
18	Normal	-1,89
19	Normal	0,67
20	T21	16,68

Algorithm:

D=-4,908+0,254*EP1+0,409*EP4+0,793*EP5+0,324*EP6+0,508*EP10+0,691*EP12

D-score 10.7 was found as optimal cut off value. The algorithm was tested with 140 test samples, which we obtain with separating samples in more aliquots. The method shows 100 % accuracy in Down's syndrome identification.

DISCUSSION

Results of our study show that described method represent fast, non-invasive, highly accurate and cost-effective approach for detecting trisomy 21 and can be performed already in the twelfth week of pregnancy. For all those reasons we could say that analysis of DMR regions of foetal DNA from maternal blood represents a promising screening method for Down syndrome and other chromosomal abnormalities and might be used in future in routine clinical practice. Such patient management would significantly decrease number of unnecessary amniocentesis and of unnecessary amniocentesis (or other invasive methods) and thus number of abortions that are caused by invasive diagnostics.

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Abstract

Catalogue of microRNA genes silenced by DNA methylation in cancer: facilitating development of novel biomarkers

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MicroRNAs (miRNAs) are small non-coding RNAs (19-25 nucleotides in length) that post-transcriptionally regulate the expression of target mRNAs and it is estimated that they regulate approximately two thirds of human genes. MicroRNA genes themselves are regulated through epigenetic mechanisms, such as histone modifications and/or DNA methylation of CpG islands. Aberrant CpG island methylation patterns are frequently associated with cancer and thus researching miRNA genes' DNA methylation is a topic of increased research interest. Due to the sheer quantity of available information, a central and up-to-date catalogue of miRNAs regulated by DNA methylation is needed, which would integrate incomplete and fragmented results from various studies. We integrated data from 149 articles and generated a catalogue of 179 miRNA genes regulated via DNA methylation in 36 cancer types with 648 entries. From the total of 2588 known mature miRNA 14.5% (179/2588) miRNAs are epigenetically regulated by methylation. 44.7% (80/179) of miRNA genes were shown to be methylated in at least two cancer types; interestingly, *hsa-miR-34b* and *hsa-miR-34c* were found to be silenced in 24 and 21 cancer types, respectively. The other 55,3% (99/179) miRNA genes regulated by methylation were found to be specific for a certain type of cancer and therefore represent specific biomarker potential. For example, *hsa-miR-191* was found to be specific for hepatocellular cancer and to promote epithelial-to-mesenchymal transition by targeting *TIMP3*. Because specific miRNAs have diagnostic and prognostic potential, the systematically assembled catalogue of epigenetically regulated miRNAs will guide researchers in their miRNA-related research.

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ABSTRACTS of POSTERS

POPULATION GENETICS

Sarah Breinig, **Laura Steinmeyer**, Andreja Ramšak

Phylogenetic relationships within family Pelagiidae (Scyphozoa, Cnidaria)

Aleksandra Šakanović, **Maša Korošec**, Zdravko Podlesek, Darja Žgur-Bertok

Expression and activity of the *Escherichia coli* genotoxin Usp

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Abstract

Phylogenetic relationships within family pelagiidae (scyphozoa, cnidaria)

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Cnidaria is an old phylum of morphologically very simple animals with common feature cnidae (stinging cells). Lack of morphological features promotes re-description of many species and changes in their taxonomic ranking. One of intriguing genus is *Chrysaora* (Pelagiidae) currently contains 15 species, three of them described in last few years. *Chrysaora* spp. are very variably and individual coloration of the medusae complicated their morphological identification. Phylogenetic relationships were studied among species in the family Pelagiidae using several loci on mitochondrial (COI, 16S, 12S) and nuclear DNA (28S and ITS regions) and include species from genera *Pelagia*, *Sanderia* and *Chrysaora* as ingroup and *Cyanea* sp. as outgroup. Loci were amplified using conserved primers for invertebrates (Folmer et al., 1994, Simon et al., 1991, Zakšek et al., 2007). The length of gapless alignment was 655 bp for COI, the length of 16S rDNA was 639 bp and part of 12S was 410bp. The highest haplotype diversity was at 16S (h=0,933, π=0,004). The model of evolution was calculated for each set of data and used for phylogenetic inference. All loci give congruent results for *Chrysaora hysoscella*, while some other species from this genus have doubtfull position on phylogenetic tree. The results revealed that all markers have good resolution and also COI can be used as well for »barcoding« purposes in scyphozoan species.

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Abstract

Expression and activity of the *Escherichia coli* genotoxin Usp

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Escherichia coli is a membrane of the gut microbiota however, pathogenic strains that produce virulence factors provoke intestinal and extraintestinal infections. The most frequent extraintestinal infections are urinary tract infections, neonatal meningitis, osteomyelitis, and bacteremia/sepsis. *E. coli* provoked bacteremia is a major health care concern, in terms of lives lost and health care costs. Recently, genetoxins that damage host DNA have been identified among *E. coli* strains namely, colibactin, the cytolethal distending toxins and more recently, Usp (uropathogenic specific protein)[1,2]. These toxins have received much attention as their activity enhances the risk of tumorigenesis. Investigations of pathogenic *E. coli* strains revealed a high prevalence of *usp* sequences among strains from patients with pyelonephritis, prostatis, urosepsis, as well as from patients with ulcerative colitis. Usp is encoded on an approximately 4 kb long pathogenicity island, Pai*usp,* with three small downstream open reading frames, designated *imu1-3* (formerly designated *orfU1-3*) that protect the producing cell against the nuclease activity of Usp[3]. Here isolation of the Usp protein and its activity against the Mono Mac 6 (MM6) human monocytic cell line and the human pulmonary epithelial cell line A549 is described. Further, to detect expression of the *usp* gene a *usp-gfp* gene fusion was prepared and low level expression observed in individual bacterial cells. Activity of the Usp-Gfp fusion protein on human cell lines was also assayed.

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ABSTRACTS of POSTERS

GENOMICS

Alenka Baruca Arbeiter, Jernej Jakše, Dunja Bandelj

Development and characterization of novel microsatellite markers from ests of developing olive fruit (*Olea europaea* l.)

Klemen Hrovat, Nejc Draganjec, Marjanca Starčič Erjavec

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Genetic variability of neurotransmitter pathways in treatment resistant schizophrenia

Nina Vesel, Maruša Vadnov, Darja Žgur-Bertok, Marjanca Starčič Erjavec

Intestinal microbiota of bears and cattle as a potential zoonotic source of extraintestinal pathogenic *Escherichia coli*

Luka Zupančič, Matevž Zorec, Lejla Pašić

Are subterranean microbes true cave dwellers? – a survey on microbial habitability

Sabina Žalig, Maja Rupnik

Gut microbiota and *Clostridium difficile* infection

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Abstract

Development and characterization of novel microsatellite markers from ESTs of developing olive fruit (*Olea europaea* l.)

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The olive (*Olea europaea* L.) is one of the most ancient domesticated fruit tree and oil producing crop of the Mediterranean basin. Despite its historical, ecological and economic importance, the first EST (expressed sequence tag) transcripts from developing olive fruits were published as late as 2009. There are only eight available EST-SSR markers for olive at the moment. In this study we present a new set of 29 EST-SSR primer sequences in olive generated from EST sequences which were the result of olive transcriptome project which included construction of normalized cDNA library from developing fruits of variety 'Istrska belica' and has been conducted by Slovenian research team. EST-SSR sequences were identified by Perl script MISA and Primer3 software was used to design the primers. Novel markers were tested for genotyping on a set of eight olive varieties. A total of 116 different alleles were detected and the number of alleles per locus varied from two to seven, with an average of 4 alleles. The observed heterozygosity ranged between 0.125 and 1.0, with an average of 0.698. Polymorphic information content (PIC) was from 0.110 to 0.795 and classified twenty-two loci as informative markers (PIC > 0.5) and eleven loci as suitable markers for gene mapping (PIC > 0.7). 29 new EST-SSR markers identified in our study not only provide a tool for diversity analysis; they may helpful to localize genes involved in agronomic and productive traits and therefore play a significant role in marker assisted selection and construction of genetic maps.

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Abstract

A tool for the Fisher's exact test

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Fisher's exact test (Fisher, R.A., 1922) is a frequently used statistical method, also in the analysis of biological data. It runs on 2 × 2 contingency table and is used to test statistically whether there is any relation between two tested categorical variables. Null hypothesis in Fisher's exact test says that there is an association (correlation) between both categorical variables, and the alternative hypothesis says that there is no association (correlation) between both tested categorical variables. With the Fisher's exact test we obtain *P*-values (probability). A *P*-value <0.05 confirms that the null hypothesis is true.

An example of a tool for Fisher's exact test is available on the link: http://www.langsrud.com/fisher.htm. The problem appears with big data. Testing big data manually with the Fisher's exact test is very time consuming, further more the chance of making mistakes is great. Because of the described problems, we have decided to develop a tool, which will upgrade Fisher's exact test. In our tool, the researcher needs to upload the Excel file with the data to the server through the user interface (specified for of the Excel file). The tool then automatically calculates all possible comparisons among the given data with the Fisher's exact test and gives as output an Excel file, with results of all comparisons (with related information of the input data) and *P*-values. The researcher just reviews all of the obtained *P*-values to see statistically significant correlations among the given data.

The tool is available via the link: www.fisher.genialis.com

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Abstract

Visualizing BLAST alignment results

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BLAST is a well known bioinformatics algorithm and software suite for comparing biological sequences – finding alignments. In most cases, users use the BLAST program running on the NCBI (http://blast.st-va.ncbi.nlm.nih.gov/Blast.cgi), which can output its results in a wide variety of text-based formats. However, the information obtained by the NCBI BLAST is dense and on first sight doesn't give the user detailed information about found alignments. NCBI's online BLAST is able to summarize the results in a simple graphical way of obtained hits. By clicking on the obtained hit, user is redirected to hit's detailed view in the alignments section. To be able to see the annotated sequence, user has to use a further link to the GenBank nucleotide database. The described process is quite time consuming.

In the need of a better user experience, we tried to stitch together information present in BLAST result files with detailed annotations obtained from GenBank, represented in a clear and intuitive graphical way. Our visualization offers obtained alignment hits plotted against the query sequence, accompanied with all details extracted from GenBank for each alignment. This includes locations on query and hit sequence and locations of alignment features, such as gene, RBS and CDS positions. For data extraction, we used Python programming language with associated Biopython library, offering access to NCBI's services. Visualization is done by JBrowse, an open-source genome browser, which offers additional interactions, such as highlighting, zooming and moving along the sequence.

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Abstract

Bioinformatics analysis of regulatory features of MyD88, an adaptor protein of innate immunity, as a basis for efficient modification of its expression.

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Adaptor protein MyD88 has a pivotal role in TLR and IL-1R signalling and is involved in mediating excessive inflammation. It leads to activation of the transcription factors NF-κB, AP1, CREB, IRF or STAT3.[1] Signalling via MyD88 is very important in some infectious diseases. Furthermore, it has been associated with suppression or enhancement of tumour formation.[2] For these reasons, MyD88 has become a subject of intense investigations in last few years. However, most studies are focusing on the mechanisms of actions and only few on regulation of MyD88 gene expression.[3,4] Thus I focused on finding regulatory regions of this gene, using experimental and prediction data in various bioinformatics databases.[5,6,7,8] I examined 2922bp-long sequence that covers the entire segment between MyD88 and its neighbouring gene ACAA1 and the first 1683bp of the 5' end of the MyD88 gene. Regulatory features analysed were sites of transcription factor binding, open chromatin, histone modifications, RNA polymerases binding, CpG islands, TATA-box, genetic variability or polymorphisms SNP, conserved sequence between species and within the human genome. After analysing them I used a computer program Microsoft Excel to compute weighed values to identify regulatory features that overlap. The results showed that the core promoter of MyD88 is most probably located between 38.138.452 and 38.139.036bp on the 3rd chromosome, and that the likely shared promoter between MyD88 and ACAA1 is located between 38.137.708 and 38.138.452bp. Furthermore, the core promoter region of MyD88 was located within the 1st exon of this gene, which is rarely observed In future, result of this study could be used to affect the expression of the gene by targeting regulatory elements using TALEN or CRISPR/Cas9 tehnology. For these methods we need at least 15bp- long target sequence. Therefore I suggested tree regions in the core promoter with highest overlapping regulatory features for further research. Furthermore, the results of BLAST genome showed that a cis-regulatory region may be located between 38140079 and 38140161bp that could also be explored for perturbin expression of Myd88l. Beside that I also propose the analysis of region between 38.137.754 and 38.137.774bp, because it surrounds SNP rs4988453 for which it has been experimentally shown to affect MyD88 transcription.[4] In summary, results of this diploma provide tools and data for future basic research in the area of MyD88 gene expression regulation as well as for applied research in biotechnology and clinical medicine.

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Abstract

Genomic analysis and subtyping of the *Escherichia coli* pathogenicity island encoding the genotoxin Usp and associated immunity proteins

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Escherichia coli is a commensal of the gut however, particular strains can provoke intestinal and extraintestinal infections due to production of virulence factors. Recently, *E. coli* strains that produce genotoxins have received much attention as chronic exposure to DNA damaging agents may provoke genome instability that enhances the risk of tumorigenesis[1]. Pathogenic E. coli strains can harbor two or more pathogenicity islands (PIs) that encode multiple different virulence determinants[2-5]. This study focused on a small pathogenicity island encoding a genotoxin, the uropathogenic specific protein (Usp) and associated Imu proteins that protect the producer against its nuclease activity[1]. We performed phylogenetic analysis on nucleotide and amino acid sequences of the *usp* and *imu* genes, originating from *E. coli* (26 sequences) and *S. bongorii* (2 sequences). We showed that in all analyzed genome sequences the pathogenicity island is inserted in the intergenic region between the *aroP* and *pdhR* genes. Further, we showed that classification based on the order of the *imu* genes[6] corresponds with clustering of *usp* and *imu* sequences. We propose four subtypes based on similarities in the C-terminal nuclease regions of *usp*. One subtype was restricted to *S. bongorii* strains and three others to which 11, 11 and 4 of the analyzed *E. coli* genomes , respectively, were assigned, with the last one being newly defined.

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Abstract

Use of glasso method to penalize small relationships in genomic prediction

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Prediction of genotypic values is important in the area of animal and plant breeding for selective breeding to improve economically important traits as well as in the area of human genetics for the prediction of phenotypes for preventive counselling and medical treatment. In the past for the prediction only the information from pedigree was used, nowadays is more established prediction of genotypic value based on the SNP markers[3, 6]. The literature describes that pedigree information matrix has sparse links within the pedigree based on expected inheritance, when on the other hand genomic relationship has dense links beyond available pedigree based on realized inheritance[1, 4]. Our aim is to set up genomic relationship matrix based on penalizing the effect of smaller relationship coefficients and to evaluate prediction accuracy. We used GLASSO method[2, 5] to minimize the importance of small relationship coefficient. GLASSO with lasso penalization helps us to estimate this inverse of variance- covariance matrix. We simulated data of 1000, 10000 and 100000 SNP markers and evaluated the prediction accuracy with calculating the correlations between true breeding value and estimated breeding values for each regularization parameter. Based on the results of simulated data we are not yet able to confirm that distant relationships are not important for the prediction of genotypic values, we need to verify simulation results on real data. The developed method for setting up genomic relationship matrix with the appropriate modelling of recombination and segregation processes represent an important contribution to the development of science of predicting genotypic values.

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Article

Genome-wide *in silico* screening (GWISS) for genetic variability of microRNA genes in cattle

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MicroRNAs (miRNA) are a class of non-coding RNAs important in posttranscriptional regulation of their target genes. Regulation requires complementarity between the target mRNA and the miRNA region responsible for their recognition and binding, also called the *seed* region. Previous studies have shown that genetic variability of miRNA genes (miR-SNP) has an impact on phenotypic variation and disease susceptibility in human and mice. Polymorphisms in miRNA genes could therefore represent biomarkers for phenotypic traits important also in livestock. However, the genetic variability of miRNA regulome in livestock has not been systematically investigated. Therefore we updated our previously developed tool miRNA SNiPer to the version 4.0 and performed genome-wide *in silico* screening (GWISS) of cattle genome to identify polymorphisms residing within the miRNA genes. Out of 808 currently known pre-miRNA regions in cattle 741 were polymorphic. Among them 452 had polymorphic mature regions including 278 miRNAs with polymorphic *seed* regions. In 61 cases *seed* regions had consecutive polymorphisms; multiple nucleotide polymorphisms (MNPs), which comprise from two to six nucleotides. Since several miRNA gene polymorphisms have an unknown validation status we selected *bta-mir-2313* gene comprising *seed* SNP for experimental validation and confirmed three additional SNPs within its pri-miRNA region. The update of the miRNA SNiPer tool and collected polymorphisms of cattle miRNA genes will serve researchers as a starting point in designing projects to examine phenotypic effects miRNA genetic variability genes in cattle.

INTRODUCTION

MicroRNAs (miRNAs) are small non-coding RNAs, usually ~22 nt in length. They are important regulators of gene expression, by binding to different target gene region they repress or activate translation (1). MicroRNA gene transcripts, also termed primary miRNA (pri-miRNA), are cleaved by Drosha enzyme to produce hairpin-shaped precursor miRNA (pre-miRNA). Dicer enzyme cleaves pre-miRNA, the result of which is double-stranded mature miRNA. One of the strands becomes the lead strain and participates in gene expression regulation. MicroRNA function is based on complementary pairing between miRNA and its target gene. The region, crucial for accurate miRNA-target binding also termed the seed region is positioned at 2-7 or 8 nucleotides within mature miRNA region. Genetic variants within miRNA genes (miR-SNPs), miRNA regulatory region (miR-rSNPs), miRNA targets (miR-TS-SNPs) and miRNA silencing machinery (miR-SM-SNPs) have been associated with phenotypic changes and disease development in human (2-4). Additionally, polymorphisms of miRNA targets have also been associated with phenotypic traits in cattle (5). However, the genetic variability of miRNA regulome in cattle has not been systematically investigated. Therefore we have updated our previously developed bioinformatics tool miRNA SNiPer tool (6, 7) with the latest database releases for the search of miRNA polymorphisms and created a catalog compiling polymorphisms within mature miRNA seed regions in cattle. We chose highly polymorphic *bta-mir-2313* for sequencing and validation of polymorphisms.

MATERIALS AND METHODS

Update of miRNA SNiPer tool

Online tool miRNA SNiPer for the search of miRNA polymorphisms has been updated to the version 4.0. The tool miRNA SNiPer integrates data from miRBase version 21, Ensembl 76 and TargetScan 6.2. It allows identification of miRNA polymorphisms in 15 animal species (http://www.integratomics-time.com/miRNA-SNiPer).

We have collected polymorphic miRNA genes in cattle using the updated version of the miRNA SNiPer tool and the list of known cattle miRNA genes obtained from miRBase 21. MicroRNA genes comprising multiple nucleotide polymorphisms (MNPs) within seed miRNA regions in cattle were identified. The miRNA gene list was supplemented with information regarding miRNA host gene, obtained from miRBase database. Information regarding pre-miRNA location, polymorphism ID, detail and location was obtained from miRNA SNiPer 4.0 tool.

Development of *bta-mir-2313* markers

Cattle samples (n=152) were obtained from the National progeny test for Slovenian Simmental cattle. DNA from semen of sires was extracted using DNeasy Blood & Tissue DNA extraction kit (Qiagen, Düseldorf, Germany). Polymerase chain reaction (PCR) primers were selected using Primer 3 tool (http://frodo.wi.mit.edu/): bta-mir-2313-F: 5'-GCACAGACTCTCAGCCACTG-3', bta-mir-2313-R: 5'-CTGACTGAGGCTCTCGCTCT-3' comprising pre-miRNA and flanking regions of the miRNA gene. Conditions for the PCR were: 94°C for 10 min, 30 cycles of 94°C for 1 min, 63°C for 1 min, 72°C for 1 min followed by a further 10 min extension at 72°C. The PCR products were purified using Exonuclease I (*Exo*I) and shrimp alkaline phosphatase (SAP) (both Fermentas, Vilnius, Lithuania), followed by the sequencing reaction assayed on the capillary electrophoresis using ABI3130xl (Applied Biosystems, USA).

RESULTS

In this study we have updated miRNA SNiPer tool for the search of polymorphisms within pre-miRNA regions in 15 animal species, performed genome-wide *in silico* screening (GWISS) of cattle genome to collect polymorphic miRNAs, created a catalog of MNPs within mature miRNA *seed* regions and performed experimental validation of polymorphisms within pre-miRNA regions of *bta-mir-2313*.

Update of miRNA SNiPer tool

We have upgraded miRNA SNiPer tool to the version 4.0. The current version enables the search of polymorphisms in 15 species; 14 vertebrate and one insect: human, mouse, zebrafish, chicken, cow, dog, horse, orangutan, pig, platypus, rat, sheep, tetraodon, zebra finch and fruitfly.

Polymorphisms within miRNA seed regions in cattle

We performed GWISS of the cattle genome for polymorphisms residing within pre-miRNA regions. Out of 808 currently identified miRNAs in cattle there were 741 polymorphic pre-miRNA genes**.** The most polymorphic pre-miRNA in cattle is *bta-mir*-212, which comprises 44 polymorphisms, including a MNP in mature miRNA *seed* region. Among 741 polymorphic pre-miRNAs in cattle, there are 452 miRNAs with polymorphic mature regions, which include 278 miRNAs with polymorphic *seed* miRNA regions. Out of 278 polymorphic bovine mature miRNA *seed* regions, there are 61 *seed* regions with two or more consecutive polymorphisms, referred also as multiple nucleotide polymorphisms (MNPs). Those 61 miRNAs include 148 polymorphisms, forming two or more consecutive polymorphisms: 56 dinucleotide polymorphisms (DNPs), including two cases of

consecutive SNP and indel, three triple nucleotide polymorphisms (TNPs), four four-nucleotide polymorphisms, one five-nucleotide polymorphism and one six-nucleotide polymorphisms mature miRNA *seed* regions. The longest regions with consecutive polymorphisms are located within *bta-mir-763* and within *bta-mir-346* in whith five and six polymorphic nucleotides within mature miRNA *seed* region, respectively (Figure 1). Out of 61 collected polymorphic miRNA genes comprising MNPs, there are 38 intergenic and 23 located within introns of protein-coding genes. Additionally, intronic miRNA *bta-mir-1839* overlaps small nucleolar RNA *SCARNA15* gene.

bta-miR-346 **Mature:** 41329108-41329130 **Seed:** 41329123-41329129 from TargetScan GGUCUCUGUGUUGGGCGUCUGUCUGCCCGC AUGCCUGCCUCUCUGUUGCUCUGAAGGAGGCA GGGGCUGGGCUUGCAGCUGCCUGGGCAGAGCGG	intergenic	28:41329055-41329149[-]	rs476404494 rs437163819 rs455988115 rs474527328 rs441539028 rs459876561	SNP (G > C) SNP (C>A) SNP (A>G) SNP (G>C) SNP (A>C) SNP (C>A)	41329124 41329125 41329126 41329127 41329128 41329129
bta-miR-763 **Mature:** 48164068-48164089 **Seed:** 48164082-48164088 from TargetScan AAUGAUGGAGGUGCAGGCAUUUCCUGAGGAUUA AUGACCAGCUGGGAGGAACCAGUGGCCCUU GGCUCUGCCUCCCAGCCAGCCAUUAAUUCCGA GGAAAUGUCUUUUGCUGAGGUCGUU	*HMGA*, intronic	5:48164007-48164126[-]	rs433455703 rs456821331 rs476820905 rs435813492 rs455946744	SNP (A > C,G) SNP (G>C) SNP (C>G) SNP (T>C) SNP (G> C,T)	48164084 48164085 48164086 48164087 48164088

Figure 1: Cattle miRNA genes bta-mir-346 and bta-mir-763 comprising the longest MNPs within mature seed regions.

Development of bta-mir-2313 markers

We have selected *bta-mir-2313* for experimental validation of polymorphisms based on various information in genomic browsers - it includes polymorphisms in both 3' and 5 seed regions' and is located within the host gene *Gram containing domain 1B (GRAMD1B)*. We have sequenced 152 cattle samples and confirmed one pre-miRNA SNP, rs41761413 and three SNPs within pri-miRNA flanking regions, rs41761412, rs41761414 and rs41761415, which all have unvalidated status in the dbSNP database. Polymorphism rs41761412 has a poly-allele substitution G>T>A.

DISCUSSION

The updated version of the miRNA SNiPer tool integrates latest releases of genomic databases and enables search for polymorphic miRNA regions in animals, including livestock species. Since the amount of genomic data is rapidly increasing, regular updates of bioinformatics tools are essential. As shown in Figure 2, 12 % of total miRNA in cattle were found to be polymorphic using the miRNA SNiPer tool version 3.0, however 91% of known miRNA genes were identified to be polymorphic using the version 4.0 of miRNA SNiPer. Additionally, the percentage of polymorphic seed regions increased as well, from 2.2% to 34.4%.

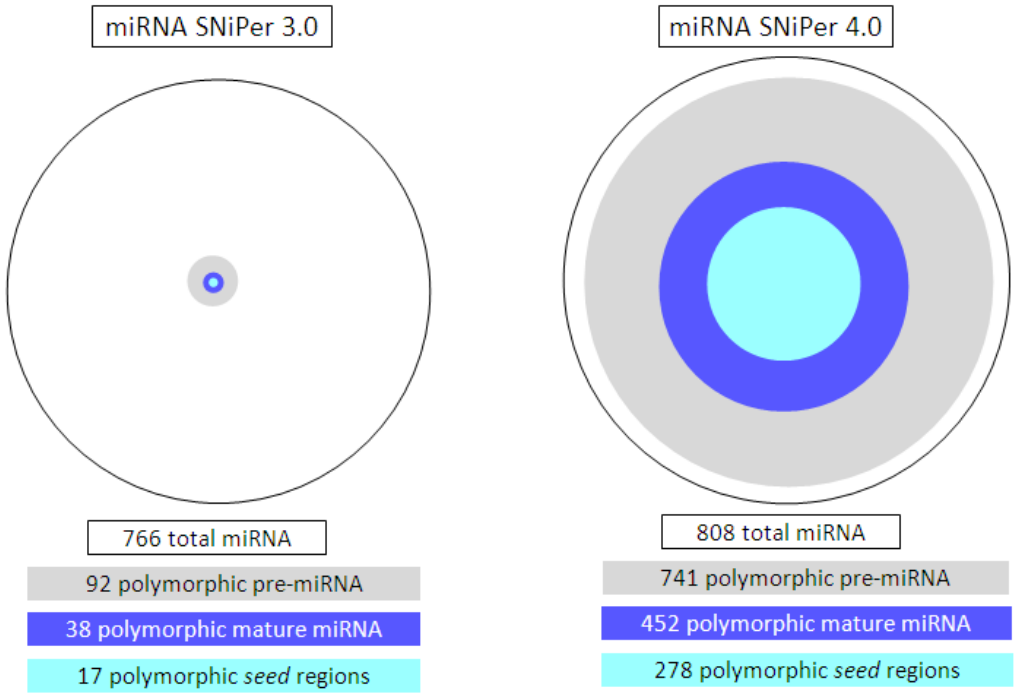

Figure 2: The comparison of polymorphic miRNA regions revealed using two versions of the miRNA SNiPer tool.

This result is unexpected since miRNA *seed* regions were considered to be conserved (8). However, several polymorphisms still have unvalidated status and need to be experimentally confirmed as they might be a result of sequencing errors. Alternatively, it is possible that a fraction of miRNAs in the cattle genome are redundant having functionally similar miRNA elsewhere in the genome and hence are in the process of accumulating mutations because of these back-up molecules. MicroRNA SNiPer is useful for miRNA research in various species, while currently it is the only bioinformatics tool that allows identification of miRNA polymorphisms not only human, but 15 different animal species.

Using miRNA SNiPer tool we have collected polymorphisms residing within miRNA seed regions and information regarding their host genes in cattle. Some interesting genomic hot spots have been revealed, for instance miRNA genes overlapped several protein-coding host genes as well as other ncRNA genes; *bta-mir-1839*, overlapped small nucleolar RNA (snoRNA) gene *SCARNA15*. Polymorphisms residing within such genomic overlap could affect the processing and function of both; miRNA, as well as snoRNA. Additionally, *bta-mir-346* with six and *bta-mir-736* with five consecutive polymorphisms in seed region have been identified; however none of these polymorphisms have been validated or had assigned minor allele frequency. Further analysis should be performed to determine, whether these polymorphisms occur simultaneously or individually. These seed MNPs affect the change of miRNA seed regions and consequently miRNA function, while such miRNA would regulate different set of target genes.

In conclusion, miR-SNPs may have a large effect on phenotypic traits and disease association. Therefore, miRNA SNiPer tool and collected data enabled prioritization of polymorphisms, which present potential novel biomarkers in animal breeding. Both, the updated miRNA SNiPer tool and the catalog provide latest information regarding miRNA polymorphisms in cattle and they are supplemented with relevant data for further analysis. Our results could help toward discovering new miRNA biomarkers, which could be used for more effective marker assisted selection.

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Abstract

Genetic diversity and distribution of hydrolase genes in cave environments as depicted by analysis of available genomic and metagenomic sequences

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The walls of Pajsarjeva jama and other carbonate caves are frequently populated by pink microbial mats. Based on 16S rRNA gene analysis we established that these rich microbial communities are dominated by members of *Actinobacteria* and *Gammaproteobacteria*[1]. These two groups show a great versatility in adaptation to a broad range of environments. Accordingly, we became interested in how they are prepared for living in nutrient-limited cave environments. To this aim, we decided to study the distribution of hydrolase genes in published genomic sequences that were most closely related to pink microbial mat phylotypes (25 genomes), as well as in available metagenomic data (metagenome of Kartchner caverns, USA). The studied hydrolases were amylase, beta glucosidase, cellulase, chitinase, esterase, lipase and xylanase.
To obtain reference phylogeny, the amino-acid sequences of the studied enzymes were first queried against the GenBank RefSeq database using Deltablast and then aligned using MUSCLE. The best-fit model of aminoacid replacement was selected using ProtTest and the reference tree was constructed using RaxML[2] and the selected model. The obtained reference tree was used as a scaffold for the placement of short metagenomic sequences using RaxML Evolutionary Placement Algorithm and alignment created using Clustal Omega. The resulting trees provided an insight into the distribution and divergence of the studied hydrolase genes. We found that this approach represents a good starting point to sequence- and function-based approach towards discovering novel biocatalysts in the abovementioned environment.

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Abstract

Effects of ionophore coccidiostat monensin on conjugation frequencies in *Escherichia coli*

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Pathogenic *Escherichia coli* is the causative agent of colibacillosis that causes diarrhea, urinary tract infections, meningitis and sepsis in domestic animals. Until the ionophore antibiotic monensin has been introduced four decades ago as a food supplement to control coccidiosis in poultry this disease has been a major cause of poor performance and low productivity in poultry and other farm animals. Since then more animals have been medicated with ionophores than any other medicinal agents in the history of veterinary medicine. It has been recently suggested, that poultry meat is an important source of extended spectrum β-lactamase (ESBL) producing *E. coli* that can colonise human gastrointestinal tract and subsequently cause extraintestinal infections. Genes encoding ESBL enzymes and other resistance genes often reside on large conjugative plasmids. The aim of our study was to investigate whether monensin enhances the frequency of conjugation between *E. coli* and thus contributes to the spread of resistance genes. Human extraintestinal and poultry *E. coli* isolates were used as ESBL plasmid donors and the sodium azid (NAz) resitant strain J53 as the recipient. Monensin was added in various concentrations (0.01 mM, 0.1 mM and 1 mM) to the liquid conjugation medium BHI. Transconjugants were selected on LB agar plates supplemented with NAz and cefotaxim (CTX). The conjugation frequencies differed between strains and ranged from 10^{-4} to 10^{-6}. For each of the tested strains the frequency of conjugation did not differ significantly due to the addition of various concentrations of monensin. We conclude that the tested concentrations of monensin do not enhance plasmid spread due to conjugation between different *E. coli* strains.

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Abstract

Genetic variability of neurotransmitter pathways in treatment resistant schizophrenia

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Schizophrenia is a chronic disease, in which one third of patients does not respond to treatment[1]. Treatment resistant schizophrenia is a public health problem[2]. Studies have shown demographical[3], radiological[4] and neurophisiological[5] differences between the treatment responsive and treatment resistant schizophrenia patients. This study explored the influence of genetic polymorphisms of the dopaminergic receptors *DRD1, DRD2, DRD3*, 5-hydroxytryptamine 1A receptor (*5-HT1A*) and serotonergic transporter *SLC6A4*, catechol-O-metyltransferase (*COMT*), *BDNF, NOTCH4, NRG1* and *DISC1* genes on the clinical symptoms and treatment resistance in Slovenian patients with schizophrenia.

We recruited 138 schizophrenia patients. Among them 94 were treatment responsive and 44 treatment resistant. Ninety-four healthy blood donors represented our control group. Genotyping for *5-HT1A* rs6295, 5-HTTLPR, *DISC1* (rs6675281, rs821616), *NRG1* (rs3735781, rs3735782, rs10503929, rs3924999), *BDNF* rs6265, *NOTCH4* rs367398, *DRD1* (rs 4532, rs 5326), *DRD2* rs 1801028, rs 1799732), *DRD3* rs 6280 and *COMT* (rs 165815, rs4680) was performed.

The data obtained suggest that *NOTCH4* rs367398 was significantly associated with worse Positive and Negative Syndrome Scale (PANSS; p=0,001) and Global Assessment of Functioning (GAF; p=0,022) scores. Furthermore, *5-HT1A* rs6295 (p=0,007) and *5-HTTLPR* (p=0,025) influenced GAF, while *5-HTTLPR* (p=0,044) also influenced the total score on the negative subscale of the PANSS. Variants of *DISC1* rs6675281 (p=0.018) and *BDNF* rs6265 (p=0,040) were associated with higher antipsychotic doses. Only *NOTCH4* rs367398 was associated with occurrence of the treatment-resistant schizophrenia (p=0,045).

Our data indicate that *NOTCH4* rs367398 can influence clinical symptoms and treatment responsiveness in Slovenian patients with schizophrenia. *NOTCH4* rs367398, *5-HT1A* rs6295 and 5-HTTLPR can influence patients' global functioning. 5-HTTLPR was associated only with negative schizophrenia symptoms, while *DISC1* rs6675281 and *BDNF* rs6265 were associated with antipsychotic dose.

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Abstract

Extraintestinal pathogenic *Escherichia coli*

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The bacterium *Escherichia coli* (*E. coli*) is a commensal of the intestinal microbiota of warm-blooded animals, and humans. However, several studies showed that the intestinal microbiota is also a reservoir for extraintestinal pathogenic *E. coli* (ExPEC) that can, due to possession of specific virulence factors (VF), instigate extraintestinal infections including urinary tract infections, meningitis, septicaemia. That healthy humans are carriers of ExPEC is well established, yet whether animals are also a potential source of human ExPEC is still under debate. The aim of this study was to estimate the potential risk of ExPEC carriage among the intestinal microbiota of bears and cattle in Slovenia. In this study 86 *E. coli* strains isolated from faeces of bears and 89 *E. coli* strains from faeces of cattle were investigated. The following VF genes APEC-*ompT, ompT*, *iroN*, *traJ, iss* and *clbAQ* were detected with PCR and the VF gene prevalences were determined. Our results were compared with the data of the VF gene prevalences among commensal human *E. coli* strains using Fisher's exact test. This study indicates that bears as well as cattle are a potential reservoir of human ExPEC, albeit with a lower prevalence of ExPEC compared to healthy humans.

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Abstract

Are subterranean microbes true cave dwellers? – a survey on microbial habitability

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The purpose of the study was to determine the geographic distribution of bacterial phylotypes that form the core of cave microbial communities sampled in four different caves in Europe; Pajserjeva cave (SI), Altamira cave (SP), Sloup-Šošůvka cave (CZ) and Vjetrenica cave (BA). The geographic distribution of phylotypes was achieved by means of 16S rRNA analysis via MetametaDB. This online database and analytic system for investigating microbial habitability is based on the BLAST algorithm of microbial 16S rRNA with annotation of isolation source properties for each sequence. The results of the study were mostly uniform, showing that the majority of the isolated bacteria from all four caves were of soil or rhizosphere origin, while sample from one particular cave showed origins of bacteria from human skin, likely indicating a human error in the analysis. This data suggest that most bacteria found in caves are closely related to nearby ground bacteria. Particularly interesting was the finding that one of the most abundant bacteria found in all four caves, a phylotype related to *Euzebya tangerina* F10(T) (HE603178), demonstrated no significant phylogenetic resemblance (above 97% 16s rRNA identity) with any other bacteria in the MetametaDB database. This might indicate a unique bacterial genome, adapted to the life in cave environments.

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Abstract

Gut microbiota and *Clostridium difficile* infection

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Clostridium difficile, an anaerobic, sporogenic bacterium, is recognized as the main cause of infectious diarrhoea associated with hospitalization. Crucial for the development of *C. difficile* infection (CDI) is disturbance of the normal gut microbiota (ie. dysbiosis), which is usually due to treatment with antibiotics.[1] Individuals colonized with *C. difficile* have less diverse gut microbiota, with the general decrease of anaerobic bacterial species (eg. *Bacteroides, Bifidobacterium*) and an increased proportion of facultative anaerobes (eg. *Enterobacteriaceae, Enterococcus*).[2] Recent studies have not only demonstrated changes within particular groups of bacteria, but also specific microbial patterns associated with CDI. Also, several studies indicated the possibility, that some subtypes of *C. difficile* might be associated with more disturbed microbiota than others.[3,4,5]

We are developing an *in vitro* system for testing interactions between *C. difficile* and fecal microbiota. Cultivation methods are coupled with molecular approaches (PCR-DHPLC, pyrosequencing) for monitoring the impact of different subtypes of *C. difficile* on abundance, composition and functionality of fecal microbiota. First results show a slight growth reduction and minor changes in compostition of the total fecal microbiota in the presence of some strains *C. difficile*. Research is under way to further clarify the interactions between *C. difficile* and fecal microbiota.

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CLOSING LECTURE

Academic career development by bridging research with society: the experience and the results of Trans2Care project

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The Trans2Care Strategic Project has received 2.61 million Euros to achieve two objectives of the Operational Programme Italy-Slovenia 2007-2013:

i) Promote R & D and the knowledge-based economy.

ii) Improve and qualify the potential employment in the sector through coordinated education.

A group of 14 researchers have been recruited for 3 years. The researchers have perfected their technological training, providing impetus to research and cross-border collaboration and have carried out an entrepreneurial training programme. They now have skills suitable for working with industry, healthcare professionals and associations. Some of them have been permanently employed in a research environment even before the end of the project. Thanks to a good internal coordination, they have also had a very rich personal life.

AUTHOR INDEX

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