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IN COLLABORATION WITH

THE SLOVENIAN SOCIETY OF HUMAN GENETICS

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OPENING LECTURE: **B. Matija Peterlin:** 3 EUREKA MOMENTS AND A DISAPPOINTMENT	8.45 – 9.30
Chairmen: Damjan Glavač, Marjanca Starčič Erjavec **MOLECULAR BASIS OF DISEASES – SHORT LECTURES**	**9.30 – 10.45**
Tim Božič: OVEREXPRESSION OF PROINFLAMMATORY CHEMOKINES CCL5 AND CCL17 AFTER GENE ELECTROTRANSFER TO TUMORS RESULTS IN ALTERED CYTOKINE EXPRESSION PROFILE	9.30 – 9.45
Ana Dolinar: EXPRESSION OF circRNAs IN AMYOTROPHIC LATERAL SCLEROSIS AND THEIR POTENTIAL AS BIOMARKERS	9.45 – 10.00
Katarina Kouter: DNA METHYLATION – EPIGENETIC FACTOR OF SUICIDAL BEHAVIOUR	10.00 – 10.15
Tinkara Remic: INTERLEUKIN-12 GENE ELECTROTRANSFER AS AN ADJUVANT TO TUMOR CELL VACCINATION	10.15 – 10.30
Kristian Urh: INVOLVEMENT OF SULFATASE 1 (*SULF1*) IN COLORECTAL CANCER	10.30 – 10.45
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Ester Stajič: EFFICIENT TARGETED MUTAGENESIS IN CABBAGE (*Brassica oleracea* var. *capitata* L.) BY TRANSIENT CRISPR/Cas9 EXPRESSION	11.30 – 11.45
Krista Lokar: BIOGEOGRAPHY AND PHYLOGENETIC RELATIONSHIPS WITHIN GENUS *Rhizostoma*	11.45 – 12.00
Katja Molan: ARE DIFFERENT *Escherichia coli* POPULATIONS REFLECTED IN METAGENOMIC DATA?	12.00 – 12.15
Neža Pogorevc: GENETIC DIVERSITY OF DREŽNICA GOAT	12.15 – 12.30
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ASSEMBLY OF THE GENETIC SOCIETY SLOVENIA II – CONTINUATION	**14.00 – 15.00**

LECTURES

OPENING LECTURE

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3 EUREKA MOMENTS AND A DISAPPOINTMENT

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POVZETEK

V času mojega raziskovalnega delovanja sem doživel tri evreka trenutke in eno razočaranje. Prvi evreka trenutek se je zgodil ob opisu sindroma golih limfocitov (BLS), ang. “Bare lymphocyte syndrome”, bolezen kombinirane imunske pomanjkljivosti in ob kloniranju CIITA, enega od okvarjenih genov odgovornih za BLS. Preostali trije geni so RFXANK, RFX5 in RFXAP, kompleks proteinov RFX, ki veže DNA. Protein CIITA je aktivator, ki veže RFX in protein P-TEFb, sledi aktivacija transkripcije genov MFC razreda II. Presenetljivo obstajajo 4 komplementacijske skupine te redke genske bolezni. Drugi evreka trenutek je bila ugotovitev, da je transkripcija pri evkariontih lahko uravnana tudi na stopnji elongacije transkripcije. Protein Tat – transaktivator HIV se veže s TAR RNA in P-TEFb za modifikacijo RNA polimeraze II potrebne za elongacijo transkripicije in procese kotranskripcije genov virusa. Kljub temu, da je Tat eden najpomembnejših aktivatorjev transkripcije, Tat ne vpliva na iniciacijo transkripcije. Pričujoče delo je predstavljalo premik v paradigmi izražanja genov evkariontov. Naši rezultati so tudi razjasnili delovanje genskih ojačevalcev, ang. “enhancerjev”. Medtem ko se na promotorjih sestavijo prediniciacijski kompleksi transkripcije, ojačevalci z upogibanjem k promotorjem omogočijo podaljšanje prepisa z RNA-polimerazo II. Tretji evreka trenutek se je zgodil, ko smo razjasnili, kako AIRE aktivira gene antigenov, ki so sicer tkivno specifični in v priželjcu nadzorujejo osrednjo toleranco. Mutacije v AIRE vodijo k sistemski avtoimunosti, imenovani APS ali APECED. Na moji raziskovalni poti sem doživel tudi razočaranje v raziskavah pri NIH, v okviru katerih smo odkrili introne pri adenovirusih ter genih za β-globin in imunoglobuline. Vodilni raziskovalec projekta našim rezultatom ni zaupal, zato jih je zadrževal in so posledično medtem bili objavljeni enaki izsledki druge raziskovalne skupine, Roberts in Sharp.

Ključne besede: AIDS, AIRE, APECED, BLS, CIITA, HIV, P-TEFb, RFX, Tat, transkripcija

ABSTRACT

In my scientific life, there have been 3 eureka moments and a disappointment. The first eureka moment included a description of the bare lymphocyte syndrome (BLS), which is a severe combined immunodeficiency, and the cloning of CIITA, one of the defective genes. Three others include RFXANK, RFX5 and RFXAP, i.e. the RFX complex that binds to DNA. CIITA is the coactivator that binds to RFX and P-TEFb to activate transcription of MHC class II genes in cells. Surprisingly, there are 4 complementation groups of this rare genetic disorder. The second eureka moment was the observation that eukaryotic transcription can be regulated at the step of elongation rather than

initiation. The HIV transactivator Tat binds to TAR RNA and P-TEFb to modify RNA polymerase II for elongation and co-transcriptional processing of viral genes. Importantly, although it is one of the most potent transcriptional activators, Tat does not affect transcription initiation. This work represented a paradigm shift in how we think about gene expression in eukaryotes. It also explained the action of enhancers rather than promoters of genes. Whereas promoters assemble preinitiation complexes, enhancers loop to promoters and allow RNA polymerase II to elongate. The third eureka moment came when we realized how AIRE activates otherwise tissue restricted antigen genes in the thymus to direct central tolerance in the host. Mutations in AIRE lead to systemic autoimmunity, called APS or APECED. The disappointment came from work at the NIH, where we saw intervening sequences or introns in adenovirus, β-globin and immunoglobulin genes, but the PI did not believe the data and thus held up the findings until critical papers from Roberts and Sharp appeared. I will describe these events in greater detail in my talk.

Key words: AIDS, AIRE, APECED, BLS, CIITA, HIV, P-TEFb, RFX, Tat, transcription

SHORT LECTURES – MOLECULAR BASIS OF DISEASES

Tim Božič
SL1: OVEREXPRESSION OF PROINFLAMMATORY CHEMOKINES CCL5 AND CCL17 AFTER GENE ELECTROTRANSFER TO TUMORS RESULTS IN ALTERED CYTOKINE EXPRESSION PROFILE

Ana Dolinar
SL2: EXPRESSION OF circRNAs IN AMYOTROPHIC LATERAL SCLEROSIS AND THEIR POTENTIAL AS BIOMARKERS

Katarina Kouter
SL3: DNA METHYLATION – EPIGENETIC FACTOR OF SUICIDAL BEHAVIOUR

Tinkara Remic
SL4: INTERLEUKIN-12 GENE ELECTROTRANSFER AS AN ADJUVANT TO TUMOR CELL VACCINATION

Kristian Urh
SL5: INVOLVEMENT OF SULFATASE 1 (SULF1) IN COLORECTAL CANCER

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SL1

OVEREXPRESSION OF PROINFLAMMATORY CHEMOKINES CCL5 AND CCL17 AFTER GENE ELECTROTRANSFER TO TUMORS RESULTS IN ALTERED CYTOKINE EXPRESSION PROFILE

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POVZETEK

Kemokini so pokazali obetavne rezultate glede povečane infiltracije imunskih celic v tumorsko tkivo, kar kaže na njihovo potencialno uporabnost na področju imunoterapije raka. V naši študiji smo po vnosu plazmidne DNA, z zapisom za vnetna kemokina CCL5 ali CCL17, v tumorske celice preučevali vpliv na tumorske celice *in vitro* in *in vivo*. V *in vitro* poskusih smo z lipofekcijo transfecirali dve mišji celični liniji raka dojke, 4T1 in E0771, in dve mišji celični liniji raka debelega črevesja, CT26 in MC38. Test preživetja je za celične linije 4T1, CT26 in MC38 48 ur po lipofekciji pokazal več kot 90% stopnjo preživetja, za celično linijo E0771 pa okoli 80% stopnjo preživetja. Analiza ekspresije po lipofekciji je v preživelih celicah pokazala signifikantno višje izražanje CCL5 in CCL17. Vzporedna analiza ekspresije 9 drugih citokinov je pokazala rahlo povišano raven izražanja IL-6 in CXCL10 v skoraj vseh celičnih linijah po lipofekciji. *In vivo* poskus na mišjem tumorskem modelu CT26 (dovoljenje: U34401-1/2015/7) z uporabo genskega elektroprenosa po intratumorskem injiciranju plazmida z zapisom za CCL5 ali CCL17 je pokazal povečano izražanje obeh kemokinov in majhen zaostanek v rasti tretiranih tumorjev. Prihodnji poskusi bodo usmerjeni v preučevanje infiltracije imunskih celic v tretirane tumorje.

Ključne besede: kemokini, CCL5 in CCL17, genski elektroprenos, imunski odziv

ABSTRACT

Chemokines have over the years showed promising results regarding enhanced infiltration of immune cells in tumour tissue, pointing to their potential use in the field of cancer immunotherapy. We investigated the effects of gene electrotransfer of plasmid DNA encoding for proinflammatory chemokines CCL5 or CCL17 in tumour cells *in vitro* and *in vivo*. For in vitro experiments two murine breast cancer cell lines, 4T1 and E0771, and two murine colon cancer cell lines, CT26 and MC38, were transfected using lipofection. Viability of 4T1, CT26 and MC38 cells remained above 90% 48h after lipofection while viability of E0771 cells was around 80%. Expression analysis after lipofection showed significantly increased expression of CCL5 and CCL17 in the surviving cells. Concurrent expression analysis of 9 other cytokines revealed slightly increased levels of IL-6 and CXCL10 in nearly all cell lines after lipofection. *In vivo* experiment on murine CT26 tumour model (animal licence: U34401-1/2015/7) carried out by utilizing gene electrotransfer (GET) after intratumoural injection of CCL5 or CCL17 plasmids resulted in increased expression of both chemokines accompanied by minor tumor growth delay. Future experiments will be directed towards elucidating the infiltration of immune cells into the treated tumours.

Key words: chemokines, CCL5 and CCL17, gene electrotransfer, immune response

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SL2

EXPRESSION OF circRNAs IN AMYOTROPHIC LATERAL SCLEROSIS AND THEIR POTENTIAL AS BIOMARKERS

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POVZETEK

Krožne RNA (circRNA) so skupina nekodirajočih RNA z relativno visoko stabilnostjo. Nastanejo med izrezovanjem intronov in so zelo zastopane v živčnem sistemu. V vzorcih periferne krvi dvanajstih bolnikov z amiotrofično lateralno sklerozo (ALS) in osmih zdravih kontrol smo določili izražanje circRNA s pomočjo mikromrež. Od skupno 13617 analiziranih circRNA smo našli 425 spremenjeno izraženih med bolniki in zdravimi kontrolami. Izražanje smo potrdili s kvantitativnim PCR na razširjeni skupini 60 bolnikov in 15 zdravih kontrol. Ena circRNA (hsa_circ_0023919) je bila pri bolnikih manj izražena, šest (hsa_circ_0000567, hsa_circ_0005218, hsa_circ_0035796, hsa_circ_0043138, hsa_circ_0063411 in hsa_circ_0088036) pa je bilo pri bolnikih bolj izraženih. Pri štirih (hsa_circ_0001173, hsa_circ_0001876, hsa_circ_0005896 in hsa_circ_0079284) nismo opazili razlike v izražanju, prav tako pri štirih (hsa_circ_0075320, hsa_circ_0081342, hsa_circ_0038929, in hsa_circ_0058058) pa izražanja nismo mogli zanesljivo določiti. Pri hsa_circ_0073647 izražanja nismo zaznali. Diagnostično uporabnost spremenjeno izraženih circRNA smo določili s pomočjo krivulje ROC. Tri circRNA (hsa_circ_0023919, hsa_circ_0088036 in hsa_circ_0063411) so imele površino pod krivuljo (AUC) večjo od 0,950 ter specifičnost in občutljivost nad 90 %. Izražanje circRNA v levkocitih je tako možni biološki označevalec za ALS, vendar so potrebne dodatne raziskave. Raziskavo je podprla Javna agencija za raziskovalno dejavnost (ARRS) v okviru raziskovalnega programa P3-0054 ter sofinanciranja mlade raziskovalke Ane Dolinar. Raziskavo je odobrila Komisija Republike Slovenije za medicinsko etiko (številka MZ: 0120-120/2018/8).

Ključne besede: amiotrofična lateralna skleroza, circRNA, izražanje, biološki označevalec

ABSTRACT

Circular RNAs (circRNAs) are a group of non-coding RNAs with relatively high stability. They are formed during the back-splicing process and highly abundant in nervous system. We performed a microarray profiling of circRNA expression in peripheral blood samples from 12 patients with amyotrophic lateral sclerosis (ALS) and 8 age- and sex-matched healthy controls. In total, expression of 13617 circRNAs was tested and 425 of them were differentially expressed between ALS samples and healthy controls. Validation of circRNA expression was performed on a larger set of samples (60 patients + 15 controls) using quantitative PCR. One circRNA (hsa_circ_0023919) was significantly downregulated, six were significantly upregulated (hsa_circ_0000567, hsa_circ_0005218, hsa_circ_0035796, hsa_circ_0043138, hsa_circ_0063411, and hsa_circ_0088036) and four showed no difference in expression levels (hsa_circ_0001173, hsa_circ_0001876, hsa_circ_0005896, and hsa_circ_0079284). However, we were unable to reliably determine the expression levels of four

circRNAs (hsa_circ_0075320, hsa_circ_0081342, hsa_circ_0038929, and hsa_circ_0058058) and one circRNA (hsa_circ_0073647) showed no expression in tested samples. ROC curves were used to evaluate the diagnostic potential of seven significantly up- or down-regulated circRNAs. Three circRNAs (hsa_circ_0023919, hsa_circ_0088036, and hsa_circ_0063411) had area under the curve (AUC) over 0.950 and also showed high specificity and sensitivity – over 90% at the optimal threshold point. CircRNA expression in leukocyte samples is a potential biomarker for ALS, however further validation is necessary. This work was supported by Slovenian Research Agency (ARRS) under research core funding no. P3-0054 and PhD thesis grant for young researcher Ana Dolinar. The study was approved by National Medical Ethics Committee of Republic of Slovenia (approval no. 0120-120/2018/8).

Key words: amyotrophic lateral sclerosis, circRNA, expression, biomarker

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SL3

DNA METHYLATION – EPIGENETIC FACTOR OF SUICIDAL BEHAVIOUR

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POVZETEK

Na svetu vsakih 40 sekund izgubimo človeško življenje za posledicami samomora. Dejavniki, ki vodijo v nastanek samomorilnega vedenja so številni, mehanizmi pa niso v celoti poznani. Kljub temu obstajajo številni indikatorji, ki povezujejo biološke dejavnike, bolj podrobno našo genetsko zasnovo in samomorilno vedenje. V naši raziskavi smo se osredotočili na metilacijo DNA, epigenetsko spremembo dobro preučeno pri sesalcih. Metilacija citozinov lahko preko različnih mehanizmov vpliva na izražanje genov in posledično na široko mrežo povezav v celicah. Tvorili smo dve homogeni skupini moških žrtev samomora, ki so za metodo izbrali samomor z obešanjem in skupino kontrolnih oseb (te so umrle zaradi nenadne srčne smrti). Z uporabo sekvenciranja naslednje generacije smo preiskovancem določili stopnjo metilacije. Analiza je identificirala številne diferenčno metilirane citozine med obema preiskovanima skupinama, pri čemer smo pri žrtvah samomora pogosteje opazili znižano stopnjo metilacije. Z dodatno analizo smo potrdili spremenjeno stopnjo izražanja nekaterih genov pri žrtvah samomora. Rezultati naših analiz podpirajo vlogo biološke komponente ozadja samomorilnega vedenja in prispevajo k osvetljevanju problematike na visoko samomorilno ogroženi slovenski populaciji.
Za raziskavo smo pridobili soglasje Komisije Republike Slovenije za medicinsko etiko, št. 0120-625/2015-2, KME109/12/15.

Ključne besede: samomorilno vedenje, epigenetika, metilacija DNA, samomor z obešenjem

ABSTRACT

Worldwide a life is lost due to suicidal behaviour every 40 seconds. Despite numerous factors leading to the onset of suicidal behaviour, the mechanisms are not fully known. Nevertheless, there are numerous indicators that link biological factors, our genetic background, with suicidal behaviour. In our study, we focused on DNA methylation, an epigenetic modification well studied in mammals. DNA methylation can affect gene expression through different mechanisms and consequently, have an impact on various cellular pathways. To analyse the potential effect of DNA methylation we formed two homogeneous groups of male suicide victims who died by hanging and the control group (cause of death was sudden cardiac arrest). Using next-generation sequencing, we determined the level of DNA methylation rate in both studied groups. The analysis identified a number of differentially methylated cytosines between the two groups investigated, with lower levels of methylation more frequently observed in suicide victims. Additional analysis confirmed altered expression of some genes in suicide victims. The results of our analyses support the role of biological factors in association with suicidal behaviour and contribute to illuminating the problem in the highly suicidal population in Slovenia.

The study was approved by the Slovenian National Medical Ethics Committee, No. 0120-625/2015-2, KME109/12/15.

Key words: suicidal behaviour, epigenetics, DNA methylation, suicide by hanging

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SL4

INTERLEUKIN-12 GENE ELECTROTRANSFER AS AN ADJUVANT TO TUMOR CELL VACCINATION

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POVZETEK

Gensko zdravljenje lahko uporabljamo kot imunostimulatorni adjuvant k drugim vrstam zdravljenja raka. Ne-virusni način genskega zdravljenja je genski elektroprenos, pri katerem električni pulzi povzročijo prehodno prepustnost plazmaleme, kar omogoči prenos DNA v celice. V naši študiji smo genski elektroprenos plazmidne DNA z zapisom za imunostimulatorni citokin interlevkin-12 uporabili kot adjuvant k vakcinaciji z mrtvimi tumorskimi celicami, ki so delovale kot vir antigenov. Tumorsko vakcino smo aplicirali oddaljeno od tumorja v kombinaciji z lokalno radioterapijo tumorja. Namen radioterapije je bil spodbuditi imunoreaktivno stanje tumorskega mikrookolja. Učinkovitost terapije smo preverili na dveh imunološko različnih mišjih tumorskih modelih; manj imunogenem malignem melanomu B16-F10 in bolj imunogenem kolorektalnemu karcinomu CT26. Poskusi so potekali v skladu z EU direktivo (2010/63/EU) in smernicami Ministrstva za kmetijstvo, gozdarstvo in prehrano Republike Slovenije (št. dovoljenja U34401–1/2015/38). Preverili smo terapevtski učinek terapije. Poleg tega nas je zanimalo, ali naša vakcina deluje tudi profilaktično, torej vpliva na izrast tumorjev. Vakcinacija je statistično značilno povečala proti-tumorski učinek radioterapije na tumorskem modelu B16-F10 (sinergizem), vendar ne na tumorskemu modelu CT26. Preliminarni rezultati so pokazali, da vakcinacija vpliva na izrast tumorjev CT26, vendar ne na izrast tumorjev B16-F10. Rezultati nakazujejo, da ima terapevtska vakcinacija večji doprinos k lokalni radioterapiji na manj imunogenih tumorskih modelih kot je B16-F10. Nasprotno ima profilaktična vakcinacija večji potencial na bolj imunogenih tumorskih modelih kot je CT26.

Ključne besede: genski elektroprenos, IL-12, tumorska vakcina, radioterapija

ABSTRACT

Gene therapy can be used as an immunostimulatory adjuvant to other cancer treatments. A non-viral method of gene therapy is gene electrotransfer that enables the transfer of genetic material by applying electric pulses, which transiently permeabilize the cell membrane. In our study, gene electrotransfer of plasmid DNA encoding immunostimulatory cytokine interleukin-12 was used as an adjuvant to vaccination with non-viable tumor cells, which presented the source of antigens. The tumor cell vaccine was applied distantly from the tumor in combination with local tumor radiotherapy with which we aimed to enhance the immunoreactivity of the tumor microenvironment. The therapeutic combination was tested on two immunologically different mouse tumor models; the less immunogenic malignant melanoma B16-F10 and the more immunogenic colorectal carcinoma CT26. All experimental procedures were performed in accordance

with the EU directive (2010/63/EU) and with the guidelines of the Ministry of Agriculture, Forestry, and Food of the Republic of Slovenia (permission no. U34401–1/2015/38). Additionally, we investigated the prophylactic effect of vaccination i.e. the effect on tumor outgrowth in both tumor models. The therapeutic vaccination significantly enhanced the anti-tumor effect of radiotherapy in the B16-F10 tumor model (synergism), but not in the CT26 tumor model. However, preliminary data indicated vaccination before tumor induction affected tumor outgrowth in the CT26 tumor model and not in the B16-F10 tumor model. Results indicate the therapeutic vaccination has a greater contribution to local tumor radiotherapy in less immunogenic tumor models like B16-F10 in contrast to the prophylactic vaccination, which has shown greater potential in more immunogenic tumor models like CT26.

Key words: gene electrotransfer, IL-12, tumor cell vaccine, radiotherapy

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SL5

INVOLVEMENT OF SULFATASE 1 (*SULF1*) IN COLORECTAL CANCER

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IZVLEČEK

Rak debelega črevesa in danke (RDČD) sodi med najpogostejše rake na svetu. Molekularne poti, povezane z razvojem RDČD, vključujejo postopno akumulacijo mutacij, epigenetske spremembe in spremembe, povezane z izražanjem genov, kar vodi v nenadzorovano delitev celic in invazivni fenotip. Vendar pa omenjene spremembe niso prisotne pri vseh primerih, kar nakazuje na še neodkrite mehanizme, povezane z razvojem RDČD. Zato smo raziskovali *SULF1*, ki je bil predhodno s pomočjo bioinformacijske analize identificiran kot kandidatni gen v povezavi z RDČD. Vključili smo 65 vzorcev biopsij, pridobljenih od bolnikov z adenomom, maligniziranim adenomom in RDČD, z ali brez metastaz v področnih bezgavkah. Izražanje *SULF1* smo analizirali z metodo verižnega pomnoževanja s polimerazo v realnem času. Izražanje *SULF1* narašča od adenoma, kjer je izražanje znižano, proti invazivnem RDČD, kjer je izražanje zvišano. Naši rezultati nakazujejo na vlogo *SULF1* v razvoju in napredovanju RDČD.

Ključne besede: rak debelega črevesa in danke, *SULF1*, diferenčno izražanje, verižna reakcija s polimerazo v realnem času

ABSTRACT

Colorectal cancer (CRC) is one of the most common cancers worldwide. Molecular pathways involved in CRC development include stepwise accumulation of mutations, epigenetic changes, and changes in gene expression, leading to uncontrolled cell division and an invasive phenotype. However, not all cases possess these changes suggesting that there should be undiscovered mechanisms for CRC development. We therefore investigated *SULF1*, previously identified as a CRC candidate gene using bioinformatics analysis. We included 65 biopsy samples from patients with adenoma, malignant adenoma, and CRC with and without nodal metastases. We examined expression of *SULF1* using quantitative real-time polymerase chain reaction. Its expression progressively increased from adenoma, where it was down-regulated, to invasive CRC, where it was up-regulated. Our findings indicate an involvement of *SULF1* in CRC carcinogenesis and progression.

Key words: colorectal cancer, *SULF1*, differential expression, quantitative real-time polymerase chain reaction

INTRODUCTION

Colorectal cancer (CRC) is ranked as third most common cause of cancer-related morbidity worldwide [1]. CRC has been identified as the second most common cancer in women and the third most common in men[2]. At least 80% of the CRC cases are sporadic, without any significant family history related to CRC development[3].

Tumor development in CRC is discretely divided into five stages, ranging from healthy mucosa to invasive carcinoma. The majority of CRC cases develop from precursor lesions, adenomas[4]. The main molecular pathways of CRC development include the chromosomal instability pathway (CIN), the microsatellite instability pathway (MSI), and the CpG island methylator (CIMP) phenotype pathway[3, 4]. The majority of events associated with tumor development occur early, before formation of the adenoma, and there is also a wide presence of sporadic CRCs without any identified well-known genetic aberrations.

Our group previously performed a bioinformatics analysis of projects investigating mRNA expression in healthy mucosa-adenoma-carcinoma samples using microarrays. Functional analysis of identified genes revealed that many of them are related to the extracellular matrix (ECM)[5] and one of them is Human Sulfatase 1 (*SULF1)* that was investigated in this study. The ECM is a superstructure, which has a supportive role, but also delivers signals to cells, determining their behavior. The ECM is directly involved in the process of epithelial mesenchymal transition (EMT) during malignant transformation and plays a major role in the progression of cancer[6].

The aim of this study was therefore to determine whether there is any significant difference in expression of *SULF1* in the CRC development and progression: from healthy mucosa to adenoma, malignant adenoma (MA), CRC without nodal metastases and CRC with nodal metastases.

MATERIALS AND METHODS

The full workflow of the study is represented in Figure 1.

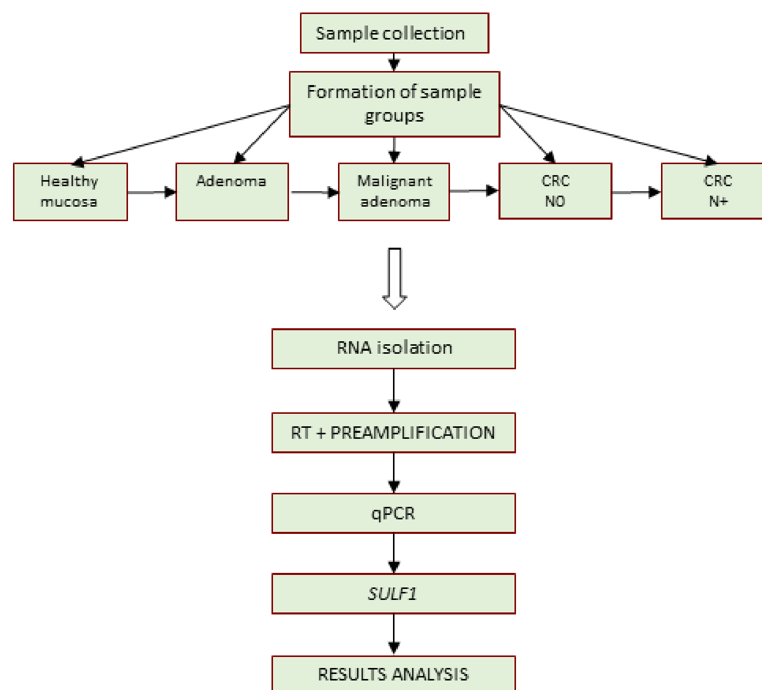

Figure 1: Workflow of the study.

Legend: CRC N0, colorectal cancer without nodal metastases; CRC N+, colorectal cancer with nodal metastases; RT, reverse transcription; qPCR; quantitative polymerase chain reaction

Sample group formation. Sample group formation was performed according to the pTNM staging of CRC[7]. Biopsy samples were collected retrospectively from the archives of the Institute of Pathology, Faculty of Medicine, University of Ljubljana. Ethical committee's approval number for the

study is 0120-88/2018/4 (22.3.2018). All biopsies were obtained during routine diagnostic and therapeutic procedures. Samples were grouped as following: healthy mucosa, adenoma, malignant adenoma (MA), CRC without lymph node metastases (CRC N0), and CRC with lymph node metastases (CRC N+). Samples of healthy mucosa obtained from resected CRC cases were used as control samples. In cases of adenoma and malignant adenoma, healthy mucosa was not available.

RNA isolation and quality assessment. RNA was obtained from formalin-fixed paraffin-embedded tissue (FFPE) tissue slides using microtome (4x 10 µm slides). RNA, including miRNAs, was isolated using AllPrep DNA/RNA FFPE kit (Qiagen) according to manufacturer's protocol. Concentration and quality assessment of the isolated RNA was performed using the spectrophotometer ND-1000 (Nanodrop) at the wavelengths 260 and 280 nm. Prior to further analyses, RNA quality was tested using reverse transcription and amplification of GAPDH (Hs_GAPDH_vb.1_SG) using SybrGreen technology.

Reverse transcription and preamplification.
Reverse transcription of the isolated RNA was performed using One*Taq®* RT-PCR Kit (NEB) using mix of random hexamers and oligo-dT primers. Preamplification of the obtained cDNA was performed using the TaqMan® Preamp Master Mix (ThermoFisher) according to the manufacturer's instructions.

Quantitative real-time PCR (qPCR). Pre-designed mixture of primers and probes (TaqMan based approach, Thermo Fisher scientific) for expression analysis of *SULF1* (Hs_00392834_m1) relatively to reference genes (RGs), *IPO8* (Hs 00183533_m1) and *B2M* (Hs 99999907_m1) was used. Prior to qPCR amplification, efficiencies were determined in triplicate reactions for each probe and for each group of samples. Rotor Gene Q machine was used for all qPCR analyses and all testing reactions were performed in duplicate. The cycling protocol was as specified: 50°C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 62°C for 1 min. Obtained ΔCq (normalized Cq of analyzed mRNAs relative to geometric mean of RGs) were used for analysis target gene expression. The fold difference in the expression of the *SULF1* gene was calculated against the healthy mucosa samples group using the ΔΔCq method, as described by Latham et al[8].

Statistics. In our experiment, mRNA expression difference was compared between CRC and healthy mucosa using ΔCq and Wilcoxon Rank test (non-parametric test for dependent samples). For comparison of relative quantification of mRNAs between independent groups (e.g. adenoma vs healthy mucosa) ΔCq and Mann-Whitney U test was used (non-parametric test for independent group of samples). All statistical analyses were performed using SPSS version 24 (SPSS Inc.). Differences in expression between groups were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

Sample groups. In total, our study included 65 samples, that were based on histologic examination divided into five groups: healthy mucosa (n=22), adenoma (n=10), MA (n=11), CRC N0 (n=10), CRC N+ (n=12).

Differential expression of *SULF1*. The fold difference in expression of *SULF1* was calculated against the healthy mucosa. Its expression progressively increased from adenoma to carcinoma. In comparison to healthy mucosa, *SULF1* expression decreased in adenoma (-35.3-fold change), whereas the most up-regulated was in CRC N+ (16.0-fold change). Figure 2 represents fold change comparison between sample groups for *SULF1*.

Table 1 summarizes results of the statistical analysis. Mann-Whitney U test was used for independent samples comparisons, e.g. healthy mucosa versus adenoma and other combinations. Wilcoxon Rank test was used for dependent samples (CRC N0/CRC N+ versus dependent healthy mucosa) as also shown in Table 1. Additional statistically significant changes were observed, with $p \le 0.05$ comparing MA versus CRC N+, $p \le 0.001$ in MA versus CRC N0, $p \le 0.001$ in adenoma versus MA, adenoma versus CRC N0 and CRC N+. CRC N0 versus CRC N+ was also statistically significant at $p \le 0.05$.

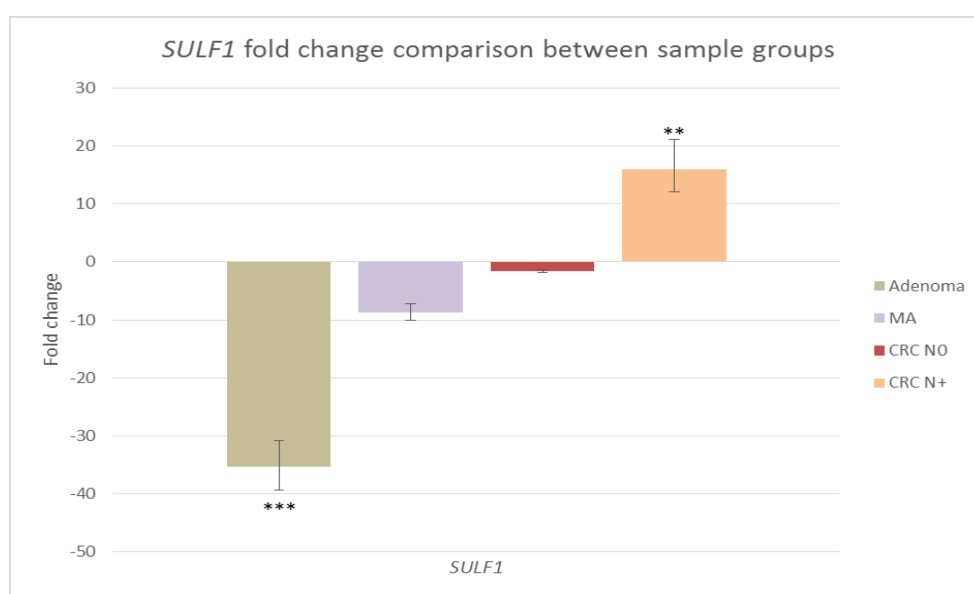

Figure 2: Fold change comparisons between sample groups for *SULF1*
Legend: MA, malignant adenoma; CRC N0, colorectal cancer without nodal metastases; CRC N+, colorectal cancer with nodal metastases, *, $p \le 0.05$; **, $p \le 0.01$; ***, $p \le 0.001$.

Table 1: Statistical results of ΔCq data comparisons using the non-parametric Mann-Whitney U test for independent samples and Wilcoxon Rank test for dependent samples

	HEALTHY MUCOSA	ADENOMA	MA	CRC NO	CRC N+
HEALTHY MUCOSA	1	≤ 0.001	0.05	/	0.004
ADENOMA		1	0.001	≤ 0.001	≤ 0.001
MA			1	≤ 0.001	0.05
CRC N0				1	0.031
CRC N+					1

Legend: MA, malignant adenoma; CRC N0, colorectal cancer without nodal metastases; CRC N+, colorectal cancer with nodal metastases

Additionally, as shown in Table 2 comparisons between the CRC N+ and CRC N0 groups with ΔΔCq data using the non-parametric Mann-Whitney U test were significant with $p < 0.001$.

Table 2: Statistical results for ΔΔCq data for CRC N+ versus CRC N0 using the non-parametric Mann-Whitney U test for two independent samples

CRC N+ vs CRC N0	**MANN-WHITNEY U TEST** ***SULF1***
Exact Sig. (2-tailed)	≤0.001

Legend: CRC N0, colorectal cancer without nodal metastases; CRC N+, colorectal cancer with nodal metastases

The results of our study show statistically significant overexpression of *SULF1* in the CRC N+ group. This is in line with some previous studies on other cancers (hepatocellular carcinoma, gastric cancer, head and neck carcinoma, pancreatic cancer, and lung adenocarcinoma)[9], but is in contrast to the down-regulation reported in the cancer cell lines derived from breast, pancreas, kidney and liver cancers[10]. However, the trend visible in Figure 2, where we observed an increase of expression from statistically significant down-regulation in adenoma to up-regulation in CRC N+, shows an interesting phenomenon for the invasive tumor phenotype, suggesting its involvement in progression of CRC. It is also important to note that *SULF1* expression has not been studied in CRC in relation to healthy mucosa-adenoma-invasive carcinoma progression.

SULF1 has been previously reported to be involved in cancerogenesis[10], including CRC[9]. It is believed to be a tumor suppressor. Target pathways of the SULF1 enzyme encoded by the *SULF1* gene include the hedgehog, Wnt and multiple heparan sulfate-dependent receptor tyrosine kinase pathways. SULF1 was shown to desulfate cellular heparan sulfate proteoglycans (HSPGs)[10]. HSPGs are complex molecules present in the cell membrane and extracellular matrix, which play vital roles in cell adhesion, migration, proliferation, and signaling pathways[9]. HSPGs therefore play vital roles in tumorigenesis, allowing cancer cells to proliferate, evade immune response, invade adjacent tissues, and metastasize to distal sites away from the primary tumor[9]. As sulfated HSPGs serve as co-receptors for many growth factors and cytokines, *SULF1* was predicted to modulate growth factor and cytokine signaling. Potential involvement in growth factor signaling and its effects on human cancerogenesis is being investigated[10]. Increased expression of SULFs in CRC has been previously reported[9]; furthermore, CRC associated genes, such as *p53*[11] and *MUC1*[12], show relationship with HSPGs. Additionally, the overexpression of SULFs in a wide range of tumors has been reported through qPCR or gene microarray. Moreover, *SULF1* was up-regulated in hepatocellular carcinoma, gastric cancer, head and neck carcinoma, pancreatic cancer, and lung adenocarcinoma[9].

In contrast, *SULF1* is reported to be down-regulated in the majority of breast, pancreas, kidney and hepatocellular cancer cell lines examined and forced expression of *SULF1* decreases cell proliferation, migration and invasion. It also promotes drug-induced apoptosis of cancer cells *in vitro*, and inhibits tumorigenesis and angiogenesis *in vivo*[10].

In conclusion, our preliminary results indicate a complex involvement of *SULF1* in CRC. It is therefore important to further research the intricacies of *SULF1* differential gene expression, which could deepen our understanding of the mechanisms of CRC development and progression.

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SHORT LECTURES – BIOTECHNOLOGY

Ajda Lenardič

SL6: *Tst* GENE ACTIVATION IN MOUSE CELL LINE USING CRISPR/dCas9-VPR SYSTEM

Ester Stajič

SL7: EFFICIENT TARGETED MUTAGENESIS IN CABBAGE (*Brassica oleracea* var. *capitata* L.) BY TRANSIENT CRISPR/Cas9 EXPRESSION

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SL6

Tst GENE ACTIVATION IN MOUSE CELL LINE USING CRISPR/dCas9-VPR SYSTEM

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IZVLEČEK

Zaenkrat ne poznamo učinkovite terapije, ki bi uspešno zdravila ali celo preprečevala debelost in sladkorno bolezen tipa 2 , hkrati pa ne bi imela sistemskih in centralnih učinkov. Tiosulfat sulfotransferaza (TST) kot eden redkih proteinov z dokazano vlogo pri ohranjanju metabolično zdravega fenotipa pri miših in ljudeh[1] predstavlja potencialno terapevtsko tarčo. Na osnovi predhodno dokazane vzročne povezave med visokim izražanjem *Tst* in nizko maso maščevja ter nizko vrednostjo glukoze v krvi[1] smo v trajni celični liniji mišjih embrionalnih fibroblastov, NIH3T3, s sistemom CRISPR/dCas9-VPR, ki vsebuje mutirani protein Cas9 brez endonukleazne aktivnosti in vezan aktivator transkripcije VPR[2], poskušali povečati izražanje tega gena. Na podlagi analize regulatornih elementov gena *Tst* smo zasnovali sgRNA in testirali njeno učinkovitost vezave na tarčno DNA preko testa cepitve z divjim tipom Cas9 v pogojih *in vitro* ter v celicah. Pokazali smo, da je izbrana sgRNA v kompleksu s Cas9 učinkovita za cepitev ciljnega zaporedja. Preko uspešnega povečanja izražanja kontrolnega gena *Pou5f1* (domena POU transkripcijskega dejavnika 1 iz razreda 5) smo z uporabo predhodno objavljene sgRNA[3] v celicah NIH3T3 dokazali tudi ustrezno aktivnost fuzije dCas9-VPR. Ko smo s kombinacijo izbrane sgRNA za gen *Tst* in dCas9-VPR poskušali povečati transkripcijo *Tst*, statistično značilne spremembe nismo opazili. Kljub temu nam je uspelo razviti reagente, postopke in bioinformatske analize, ki bodo služile kot osnova za nadaljnje poskuse manipulacije transkripcije endogenih genov s sistemom CRISPR/dCas9 za potencialni razvoj terapij preko regulacije izražanja genov brez spreminjanja genoma.

Ključne besede: debelost, sladkorna bolezen tipa 2, Tst, CRISPR/dCas9-VPR

ABSTRACT

Currently, there is no available treatment that would efficiently cure or even prevent obesity and type 2 diabetes without having systemic and/or central side effects. As one of a few proteins with proven role in maintaining metabolic health in mice and humans[1], thiosulfate sulfotransferase (TST) represents an interesting potential therapeutic target. Because higher *Tst* expression was previously demonstrated to be causal for lower fat mass/lower blood glucose levels[1], we tried to increase the *Tst* expression in mouse-derived fibroblast cell line NIH3T3 by using CRISPR/dCas9-VPR system, containing catalytically inactivated protein Cas9 with fused transcriptional activators VPR[2]. Firstly, sgRNA was designed based on our bioinformatic analyses of regulatory regions in the *Tst* locus. Selected sgRNA was found to be efficient as we demonstrated cleavage of the target DNA *in vitro* and in the cellular environment when combined with the wild type Cas9. By successful transcriptional activation of control gene *Pou5f1* (POU domain, class 5, transcription factor 1) using previously described sgRNA[3], we confirmed that dCas9-VPR fusion also worked in the NIH3T3 cells. However, significant up-regulation of the *Tst* expression by our designed *Tst*-specific sgRNA and

dCas9-VPR was not achieved. Nevertheless, we managed to develop reagents, procedures and bioinformatic analyses that will provide a good basis for further experiments to manipulate transcription of endogenous genes using CRISPR/dCas9 system and therefore for eventual development of treatments for diseases that could be treated by manipulating gene expression without the need to modify the genome.

Key words: obesity, type 2 diabetes, *Tst*, CRISPR/dCas9-VPR

INTRODUCTION

Nowadays, obesity and type 2 diabetes (T2D), often associated with it, are amongst the most widespread health disorders. Thirteen percent of the world adult population is obese and 39 % is overweight[4], while 8% suffer from type 2 diabetes[5]. Both disorders often lead to further health issues such as cardio-vascular diseases, polycystic ovary syndrome, kidney failure and certain types of cancer[6,7]. Currently, there is no available treatment that would efficiently cure or even prevent obesity and type 2 diabetes without having systemic and/or central side effects.

In 2016, increased thiosulfate sulfotransferase (*Tst*) gene expression was proven to be causal for lowering fat mass and blood glucose levels in mice as well as in humans[1]. It was also shown that *Tst* overexpression in mice leads to higher glucose uptake and improved insulin sensitivity in target tissues, while *Tst* knockout mice (*Tst-/-*) are prone to the development of glucose intolerance and insulin insensitivity. Therefore, *Tst* transcription activation could represent a very interesting therapeutic strategy for the treatment of obesity and T2D[1].

The aim of our study was to increase *Tst* expression in mouse-derived cell line NIH3T3 by using modified CRISPR/Cas9 system, known as CRISPR/dCas9-VPR[2]. In this system, Cas9 protein is replaced by Cas9 with two point mutations (H840A and D10A) which lead to the loss of its nuclease activity, making it catalytically dead (dCas9), but still able to bind to DNA. When activator domains VP64, p65(RELA) and Rta are fussed to dCas9, the resulting protein fusion is called dCas9-VPR. In combination with the appropriate sgRNA, dCas9-VPR is able to activate transcription of the target gene by recruiting transcription machinery to the promoter site.

MATERIAL AND METHODS

sgRNA design. Spacer sequence of the sgRNA for *Tst* activation was designed based on bioinformatic analysis of the *Tst* locus and its regulatory elements[8], using two on-line tools[9,10]. Selected sgRNA spacer sequence (5'-GGUAAUCAGUGCGCCCGGAG-3') is able to bind to the mouse chromosome 15, between 78406206 and 78406226 bp. sgRNA with the chosen spacer sequence was named sgRNA_Sy and ordered in synthetic form from commercial provider (Synthego).

Testing sgRNA_Sy by *in vitro* cleavage of the target DNA. A fragment of mouse genomic DNA containing sgRNA_Sy binding site was amplified by PCR using primers Tst5.F1 (5'-TCACCGGAATTGGACATTCTA-3') / Tst4.R1 (5'- GCTAAGAGCTTCGGTGCATTAC-3') and purified by QIAquick PCR purification Kit (Qiagen). The purified DNA fragment was cleaved by synthetic sgRNA_Sy (Synthego) and recombinant protein Cas9 (SIGMA-ALDRICH), following the protocol, summarized in Table 1. Results of the cleavage were analysed by agarose gel electrophoresis (1.5 % gel, 55 min, 75 V).

Table 1: Protocol for *in vitro* cleavage of the target DNA.

component	amount
10x reaction buffer (200 mM HEPES + 1mM NaCl + 1mM EDTA, pH = 6.5)	3 µl
Cas9	700 nM
synthetic sgRNA_Sy	700 nM
nuclease-free water	30 µl-V (DNA)-V(sgRNA_Sy)-V(Cas9)
20 min incubation at room temperature (RT)	
DNA (γ = 25 ng/µl)	70 nM
65 min incubation at 37 °C	
1 µl of proteinase K (Thermo Scientific) + 10 min at RT	

Testing sgRNA_Sy in cellular environment. To test sgRNA_Sy in the cells, vector EGxxFP_Sy was prepared. The vector contains target site for sgRNA_Sy, flanked by 5'- and 3'- terminal fragments of enhanced green fluorescence protein (eGFP) coding sequence that share 482 bp of homologous sequence. eGFP cannot be expressed from intact vector EGxxFP_Sy, but the expression can be achieved by the delivery of the vector to mammalian cells, followed by cleavage of the sgRNA_Sy binding site. In case of cleavage, newly formed double strand break on the vector DNA is repaired by homologous recombination, which leads to the formation of functional eGFP coding sequence. In order to test sgRNA_Sy, cells were therefore co-transfected with vector EGxxFP_Sy and another vector, containing sgRNA_Sy and Cas9 coding sequence (pX330_Sy). In case of successful cleavage, eGFP expression was expected to be observed approximately 2 days after co-transfection with these vectors. Due to much higher transfection efficiency HEK293 cells were used for the experiment.

For the EGxxFP_Sy preparation, a fragment, containing sgRNA_Sy binding site was amplified from the mouse genomic DNA by PCR with primers EGxxFP_Sy.F (5'-CAAGGATCCGGCGGTACAATGGTGC-3') / EGxxFP_Sy.R (5'- GCTGAATTCGGTGGGCGTGGCTAAGA -3') to which *Eco*RI and *Bam*HI restriction sites were added. Amplicons that hence contained *Eco*RI and *Bam*HI sites, were digested with the respective restriction enzymes and ligated to the *Eco*RI / *Bam*HI-cut vector pCAG-EGxxFP (Addgene #50716). Vector for sgRNA_Sy and Cas9 expression (pX330_Sy) was prepared by ligating the annealed DNA oligonucleotides with the sgRNA_Sy spacer sequence to the pX330-U6-Chimeric_BB-CBh-hSpCas9 vector (Addgene #42230), according to protocol[11]. Oligonucleotides, used for annealing were sgRNA_Sy.F (5'-CACCGGTAATCAGTGCGCCCGGAG-3') and sgRNA_Sy.R (5'-AAACCTCCGGGCGCACTGATTACC-3').

Testing dCas9-VPR. To test protein fusion dCas9-VPR, expressed from vector pCMV-dCas9:NLS-VPR, we tried to activate the expression of POU domain, class 5, transcription factor 1 (*Pou5f1*) that had already been successfully activated in the NIH3T3 cells by Hu et al[3]. For this experiment, the coding sequence for sgRNA_T2 designed by Hu et al.[3] was prepared by ligating the annealed DNA oligonucleotides with the sgRNA_T2 spacer sequence to the pX330-U6-Chimeric_BB-CBh-hSpCas9 vector (Addgene #42230), as described[11]. Oligonucleotides, used for the annealing were sgRNA_T2.F (5'-CACCGAACCTCCGTCTGGAAGACAC-3') and sgRNA_T2.R (5'-AAACGTGTCTTCCAGACGGAGGTTC -3'). Linear fragment, containing U6 promoter and sgRNA sequence was amplified from the prepared vector by using primers pX330.F (5'-GAGGGCCTATTTCCCATGATT-3') and pX330.R (5'-AAAAAAGCACCGACTCGGTG-3') and used for co-transfection with pCMV-dCas9:NLS:VPR.

Cell lines and culturing conditions. Cells were cultured in a monolayer in 25 cm^2 cell culture flasks (CELLSTAR) in Dulbecco's Modified Eagle Medium (DMEM) (SIGMA-ALDRICH), supplemented

with 10% fetal bovine serum (FBS) (SIGMA-ALDRICH), 1% 200mM L-glutamine and 1% penicillin/streptomycin (SIGMA-ALDRICH) at 37°C in a 5% CO_2 atmosphere.

Transfection. 24h before transfection, 5×10^5 cells were seeded in 6-well plates. All transfections were performed by Lipofectamine 3000 Transfection Kit (Invitrogen). For DNA co-transfections (pX330_Sy + EGxxFP_Sy or pCMV-dCas9:NLS:VPR + linear DNA fragment), the total mass of both DNAs used for the transfection was 2.5μg, while their molar ratio was 1:1. For pCMV-dCas9:NLS:VPR + synthetic sgRNA_Sy co-transfection, mass ratio sgRNA:DNA was 0.6:1, while a total mass was 2.5μg. For each transfection, 7μl of Lipofectamine 3000 was used.

RNA isolation and reverse transcription. RNA was isolated using RNeasy Mini Kit (Qiagen) and reverse transcribed into cDNA by using High Capacity cDNA Reverse Transcription Kit (ABI) following manufacturers' protocols.

Quantitative reverse-transcription PCR (qRT-PCR). For relative quantification of *Tst* and *Pou5f1*, qRT-PCR was performed in Viaa7 (ABI), using PowerUp™ SYBR™ Green Master Mix kit (Thermo Fisher). The primers used are listed in **Error! Reference source not found.**. The fold change in the expression of the *Tst* and *Pou5f1* between treated and untreated samples was calculated by using the $2^{-\Delta\Delta C_T}$ method, as described by Livak et al.[12]. For normalization, *Actb*, *Gapdh* and *B2m* genes were used.

Table 2: Primers for qPCR.

primer	sequence (5'->3')
mACTB.F	GTGACGTTGACATCCGTAAAGA
mACTB.R	GCCGGACTCATCGTACTCC
mB2M.F	TTCTGGTGCTTGTCTCACTGA
mB2M.R	CAGTATGTTCGGCTTCCCATTC
mGAPDH.F	AGGTCGGTGTGAACGGATTTG
mGAPDH.R	TGTAGACCATGTAGTTGAGGTCA
mTST.F	CAGCTGGTGGACTCTCGG
mTST.R	GAAGGGCATGTTGACTGAGC
mOCT4.F	GCCCTCCCTACAGCAGATCACTCACATCG
mOCT4.R	AAGGTGTCCCTGTAGCCTCATACTCTTCTCGT

RESULTS AND DISCUSSION

Testing sgRNA_Sy by *in vitro* cleavage of the target DNA. The first step was to determine whether sgRNA_Sy that had been chosen for *Tst* activation experiment by bioinformatic analysis enables DNA cleavage with wild type Cas9. Results of the target DNA cleavage *in vitro* analysed by agarose electrophoresis showed that the cleavage with sgRNA_Sy and Cas9 was successful (Fig.1). As the majority of the amplicon has been cleaved (Fig.1, lane 3), this suggests high efficiency of sgRNA_Sy.

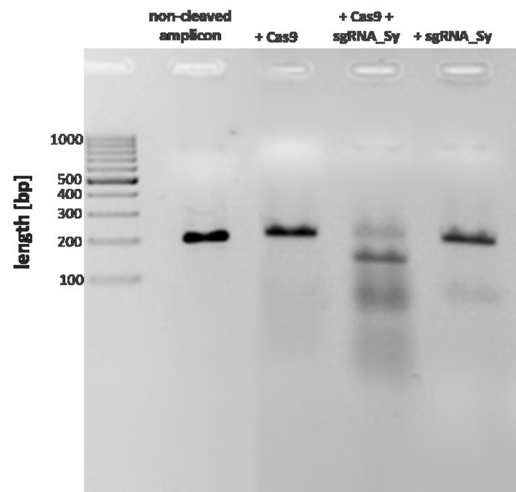

Figure 1: In vitro cleavage of target DNA by sgRNA_Sy and Cas9.

Testing sgRNA_Sy in the cellular environment. sgRNA_Sy was also tested in the cellular environment. 48 h after transfection with vectors EGxxFP_Sy and pX330_Sy, green fluorescence was observed (Fig. 2B). The number of cells showing fluorescence as well as a relatively high level of fluorescence per cell indicates that sgRNA_Sy worked efficiently also in the cell-line environment.

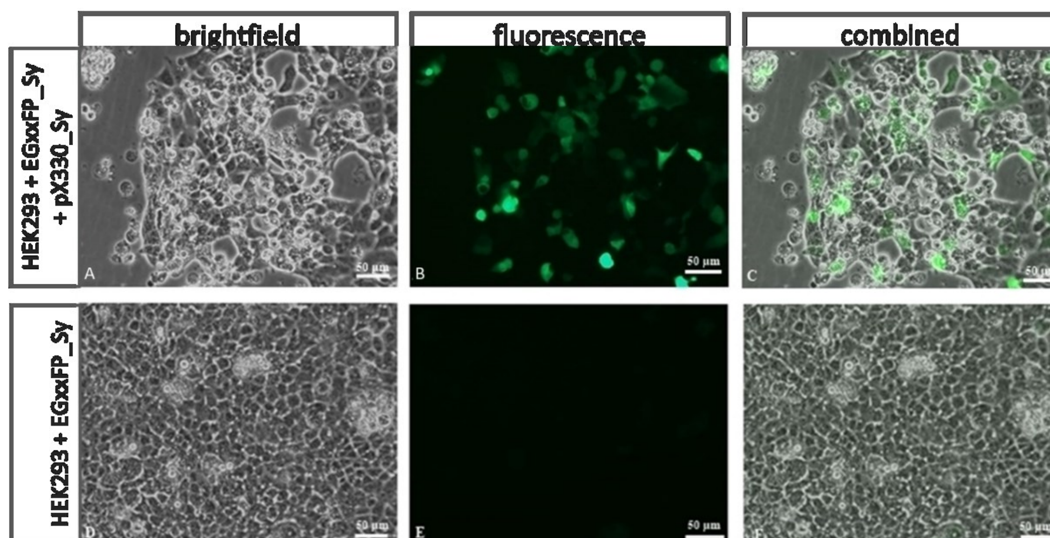

Figure 2: Fluorescence of HEK293s 48 h after co-transfection with EGxxFP_Sy and pX330_Sy.

Testing dCas9-VPR protein fusion. According to the results of the qPCR, by transfecting cells NIH3T3 with pCMV-dCas9:NLS:VPR and linear DNA sequence coding for sgRNA_T2, we were able to achieve 3.03(± 0.3)-fold activation of *Pou5f1* expression. Since this activation was higher than the one, achieved by the same sgRNA in the reference paper[3], we concluded that the second component of the activation system, dCas9-VPR, also functions efficiently in the NIH3T3 cells (Fig.3A).

***Tst* gene activation.** Since both activation components seemed to work well, we tried to upregulate *Tst* expression by co-transfecting cells NIH3T3 with sgRNA_Sy in synthetic form and vector pCMV-dCas9:NLS:VPR. No significant increase in gene expression was observed (Fig.3B). Lack of activation might be the consequence of poor transfection efficiency, suboptimal sgRNA design, lack of constitutive expression of sgRNA and/or lack of transcription factor(s) required for *Tst* expression in NIH3T3 cell line.

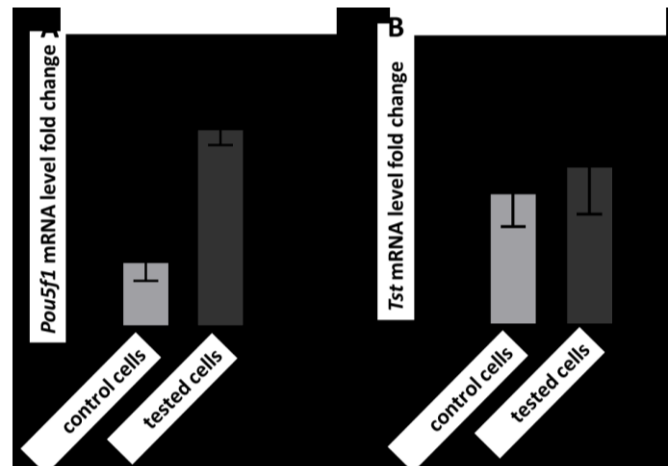

Figure 3: Fold change in Pou5f1 (A) and Tst (B) expression in tested and control cells. Results are shown as mean ±SD.

Despite unsuccessful *Tst* activation in NIH3T3, we managed to develop essential components (sgRNA and dCas9-VPR protein fusion) that showed efficient activities in separate control *in vitro* and cell-line experiments. These reagents form a basis for follow-up attempts to upregulate expression of *Tst* and potentially some other genes by the CRISPR/dCas9 system. This system provides great promise in the development of treatments for obesity, type 2 diabetes and some other diseases as this approach does not induce a double-stranded DNA break. By combining sgRNAs designed to target chosen regulatory elements, one is able to achieve robust up- or down-regulation of target gene expression by using transcriptional activators (like VPR in our study) or repressors (like KRAB domain)[13]. In comparison to conventional methods of gene therapy, modulating expression of target genes by dCas9 system does not involve a risk of insertional mutagenesis or other safety concerns. Therefore, the CRISPR/dCas9-based systems could drive development of transcription modulation-based therapies without the risk of DNA damage and modifications.

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SL7

EFFICIENT TARGETED MUTAGENESIS IN CABBAGE (*Brassica oleracea* var. *capitata* L.) BY TRANSIENT CRISPR/Cas9 EXPRESSION

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POVZETEK

Tarčno preurejanje genoma s sistemom CRISPR/Cas9 je danes najpogosteje uporabljena metoda za induciranje tarčnih mutacij. CRISPR/Cas9 je z RNA voden sistem, ki vključuje protein Cas9, ki cepi želene tarčne sekvence v genomu in s tem ustvari dvoverižne prelome. To vodi do nastanka različnih mutacij na tarčnih mestih. Metode prehodne transformacije imajo številne prednosti pri preverjanju učinkovitosti delovanja sistema CRISPR/Cas9, saj omogočajo hitro detekcijo nastalih mutacij. V naših poskusih smo uporabili dve različni metodi za prehodno izražanje CRISPR/Cas9 vektorjev: transformacijo protoplastov in infiltracijo *Agrobacterium tumefaciens*. Izbrali smo dve različni regiji v genu za centromerni protein (*cenh3)* v zelju - pomembni zelenjadnici. Gen *cenh3* je povezan z ločevanjem kromosomov in ima potencial za razvoj linije za indukcijo haploidov. Za identifikacijo induciranih mutacij na tarčnih mestih smo uporabili metodo naslednje generacije sekvenciranja (NGS). Tarčno mutagenezo smo potrdili za obe regiji in vse testirane sgRNA. Odstotki indel mutacij so se gibali od 0,07 do 9,98 % po transformaciji protoplastov in od 0,07 do 14,42 % po agroinfiltraciji.

Ključne besede: zelje, tarčna mutageneza, CRISPR/Cas9, *cenh3*

ABSTRACT

Genome editing using CRISPR/Cas9 system is nowadays the most commonly used method for induction of target mutations. CRISPR/Cas9 is a RNA-guided system with Cas9 protein, which cleaves desired target sequences in the genome and generates double-stranded breaks. This leads to the induction of different mutations at target sites. Transient transformation methods for assessment of genome editing events have many advantages, as they offer the possibility to validate the efficiency of CRISPR/Cas9-induced mutations quickly. In our experiments, we used two different delivery methods for transient expression of CRISPR/Cas9 vectors: protoplast transfection and infiltration of *Agrobacterium tumefaciens.* We targeted two different sites in the centromere-specific histone H3 *(cenh3)* gene in cabbage – an important crop plant. *Cenh3* gene is associated with chromosome segregation and has the potential for development of haploid inducer line. For identification of induced mutations at target sites, next-generation sequencing (NGS) was used. We confirmed targeted mutagenesis for both target sites and all sgRNA tested. Indel frequencies varied from 0.07 to 9.98% after protoplast transfection and 0.07 to 14.42% after agroinfiltration.

Key words: cabbage, genome editing, CRISPR/Cas9, *cenh3*

SHORT LECTURES – POPULATION GENETICS

Krista Lokar

SL8: BIOGEOGRAPHY AND PHYLOGENETIC RELATIONSHIPS WITHIN GENUS *Rhizostoma*

Katja Molan

SL9: ARE DIFFERENT *Escherichia coli* POPULATIONS REFLECTED IN METAGENOMIC DATA?

Neža Pogorevc

SL10: GENETIC DIVERSITY OF DREŽNICA GOAT

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SL8

BIOGEOGRAPHY AND PHYLOGENETIC RELATIONSHIPS WITHIN GENUS *Rhizostoma*

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IZVLEČEK

Sistematika rodu *Rhizostoma* (Cuvier, 1788) je še vedno nedorečena, pri čemer je pomembno poudariti, da še vedno niso razjasnjeni sorodstveni odnosi med vrstami *Rhizostoma pulmo* (Macri, 1778), *Rhizostoma octopus* (Linnaeus, 1788) in *Rhizostoma luteum* (Quoy in Gaimard, 1827). Zaradi preproste telesne zgradbe meduze in polipa je težko najti zanesljive taksonomske znake. Za razjasnitev filogenetskih odnosov smo pridobili nove podatke iz jedrne (28S, ITS 1 in 2) ter z mitohondrijske DNA (COI) iz vrste *Rhizostoma pulmo* ter *Rhizostoma luteum*. V analizo smo vključili sekvence *R. pulmo*,*R. luteum* in *R. octopus*. Za izračun filogenetskih dreves smo uporabili Bayesianski pristop in metodo največjega verjetja ter naredili mrežne analize haplotipov. Rezultati podpirajo delitev rodu *Rhizostoma* na tri vrste, kar je posledica biogeografskih dogajanj v preteklosti ter sedanjega vpliva Almerijsko-Oranske fronte, ki preprečuje stik med vrstami.

Ključne besede: *Rhizostoma,* molekularna filogenija, genetski označevalci, PCR

ABSTRACT

Phylogeny of genus *Rhizostoma* (Cuvier, 1788) is still uncertain and relationships between *Rhizostoma pulmo* (Macri, 1778), *Rhizostoma luteum* (Quoy in Gaimard, 1827) and *Rhizostoma octopus* (Linnaeus, 1788) are unclear. Due to the simple morphology of medusae and polyp reliable taxonomic characters are hard to find. To clarify phylogenetic relationships between the species *Rhizostoma pulmo* and *Rhizostoma luteum*, new data were retrieved from nuclear (28S, ITS1 and 2) and mitochondrial (COI) DNA. Nucleotide sequences from *R. pulmo*, *R. luteum* and *R. octopus* were included in our research. We used Bayesian inference and Maximum likelihood estimation for calculation of phylogenetic trees and haplotype network was made. Results showed a well supported division of genus *Rhizostoma* into three species due to biogeographic events in the past and recent impact of the Almeria-Oran front, which prevents inter-species contact.

Key words: *Rhizostoma*, molecular phylogeny, genetic markers, PCR

INTRODUCTION

Geological history played a significant role in distribution of populations and species. This is especially valid for the Mediterranean Sea, a semi closed sea between Europe and Africa with a turbulent geological history. One of the events with high significance was the Messinian Salinity Crisis (MSC) before 5.5 Ma and more recently the Pleistocene glaciation. Both mentioned events caused abrupt changes in the sea level and connection with the East Atlantic was interrupted several times. In present time an important biogeographic barrier in the Mediterranean Sea is the Almeria-Oran

front (AOF) which prevents migration of many pelagic species from the Mediterranean Sea to the Atlantic Ocean (Patarnello et al., 2007).

Genus *Rhizostoma* is represented by three species of rhizostomatid jellyfish which are found in the East Atlantic Ocean and in the Mediterranean Sea. Rhizostomae differs from other orders within Scyphozoa by the absence of marginal tentacles and, structure of manubrium, which forms eight oral arms with numerous mouth openings. *R. pulmo* is found in the Mediterranean and the Black Sea, *R. octopus* at the Portugal coast and in the North Sea, while *R. luteum* along the south Portugal coast, the Strait of Gibraltar and west Africa coast (Mayer, 1910). Morphological differences among species are not considerable, the main obstacle is a lack of knowledge on the nature of characters and their direction of evolution. Use of molecular markers is a well-established way to solve phylogenetic relationships between species. In this study, phylogenetic inference was made using sequences of the mitochondrial COI gene and nuclear 28S, ITS1 and ITS2 regions.

The aim of this study is to clarify relationships between species (*R. pulmo, R. luteum* in *R. octopus*) in genus *Rhizostoma* by using molecular phylogeny.

MATERIAL AND METHODS

Samples

The study included 96 individuals of *R. pulmo*, 65 individuals of *R. octopus* and 27 individuals of *R. luteum* from five biogeographic regions (North East Atlantic, West, Central and East Mediterranean and North Adriatic Sea). The final dataset consisted from sequences obtained during this study and from sequences published in previous published studies (Faleh et al., 2017; van Walraven et al., 2016, Lee et al., 2013; Ramšak et al., 2012) and deposited in GenBank. Tissue from bell margin and gonads of new individuals was preserved in 96% ethanol and frozen at -80 °C until DNA extraction.

DNA extraction, PCR amplification and sequencing

DNA was extracted using E.Z.N.A. Mollusc DNA kit (Omega, ZDA). The 28S, ITS1 and ITS2 regions and COI gene fragments were amplified.All PCRs were performed in a total volume of 50 µL with 5 µL (1-50 ng/µL) of genomic DNA, 1.25 U of Taq DNA Polymerase, 10 µM primers, 2mM dNTPs, and 2mM $MgCl_2$. Amplification of 28S region was made by touch down PCR thermal profile as follows: 3 min denaturation at 95 °C, followed by 5 cycles of 95 °C for 45 s, incremental drop from 65 to 57 °C for 80s and 72 °C for 1 min, the annealing temperature was reduced in steps of 2 °C every cycle from 65 to 57 °C, followed by 6 cycles of 95 °C for 45 s, 57 - 47 °C for 1 min and 72 °C for 1 min, the annealing temperature was reduced in steps of 2 °C every cycle from 57 to 47 °C, followed by 35 cycles of 45 s at 95 °C, 1 min at 47 °C, 1 min at 27 °C and terminated with 10 min extension at 72 °C. The thermal profile of PCR amplifications for ITS1 and ITS2 regions began with a 3 min denaturation at 95 °C, followed by 35 cycles of 1 min at 95 °C, 1 min at 48 °C, 90 s at 72°C and terminated with a 1 min extension at 72 °C. The thermal profile of PCR amplifications for COI region began with a 5 min denaturation at 94 °C, followed by 40 cycles of 30 s at 94 °C, 45 s at 55 °C, 45 s at 72°C and terminated with a 7 min extension at 72 °C. PCR products were purified and sequenced by Macrogen (www.macrogen.com, The Netherlands) using the same primer pairs as described below (Table 1).

Table 1: List of primers used in this research

Markers	Primers	Primers: 5´ to 3´	References
28S	Aa_H28S_1078	5'-GAA-ACT-TCG-GAG-GGA-ACC-AGC-TAC-3'	Bayha et al., 2010
	Aa_L28S_21	5'-GAA-CRG-CTC-AAG-CTT-RAA-ATC-T-3'	Bayha et al., 2010
ITS1	Its1	5'-GTT-TCC-GTA-GGT-GAA-CCT-GC-3'	White et al., 1990
	Its2	5'-GCT-GCG-TTC-TTC-ATC-GAT-GC-3'	White et al., 1990
ITS2	Its3	5'-GCA-TCG-ATG-AAG-AAC-GCA-GC-3'	White et al., 1990
	Its4	5'-TCC-TCC-GCT-TAT-TGA-TAT-GC-3'	White et al., 1990
COI	ScyCOIr	5’-AAA-TGT-TGG-AAT-ART-ATT-GGR-TCT-CCT-3’	Walraven et al., 2016
	ScyCOIf	5’-AAA-TGT-TGG-AAT-ART-ATT-GGR-TCT-CCT-3’	Walraven et al., 2016

Phylogenetic analysis and haplotype network

Sequences were examined and edited using Chromas Pro 2.1.8 (Technelysium Pty Ltd 2005, Australia) and aligned using AliView 1.22 (Larsson, 2014). The total data consisted of 932 bp of 28S from 53 medusae, 545 bp of ITS1 from 50 medusae, 558 bp of ITS2 from 60 medusae and 654 bp of COI from 141 medusae. Phylogeny was analysed with Bayesian inference (BI) and Maximum likelihood (ML) method. Optimal model of evolution was selected with jModelTest-2.1.10 (Posada, 2008). The best models are HKY+G for 28S, K80+g for ITS1, GTR+ G for ITS2 and GTR+I for COI. Only unique haplotypes were used for phylogenetic analyses. BI trees were made with MrBayes v3.2.6 (Ronquist, 2012). Each analysis consisted from two runs with four chains (one cold and three heated) that were run for $1x10^6$ generations and were sampled every 500 generations. ML trees were made with PhyML 3.0. (Guindon and Gascuel, 2003), where bootstrap was set to 500. Phylogenetic relationships between haplotypes were visualized in the program FigTree version 1.4.3. (Rambaut, 2009). Program PopArt 4.8.4 (Leigh and Bryant., 2015) was used for haplotype network analysis. Median joining networks for COI, ITS1 and ITS2 was constructed using default settings.

RESULTS AND DISCUSSION

Phylogenetic analysis

Phylogenetic trees constructed with BI and ML methods had very similar topology and high branching support. Due to similar topology, we presented only trees made with BI, together with relative support at branching points calculated with ML method (Figure 1).

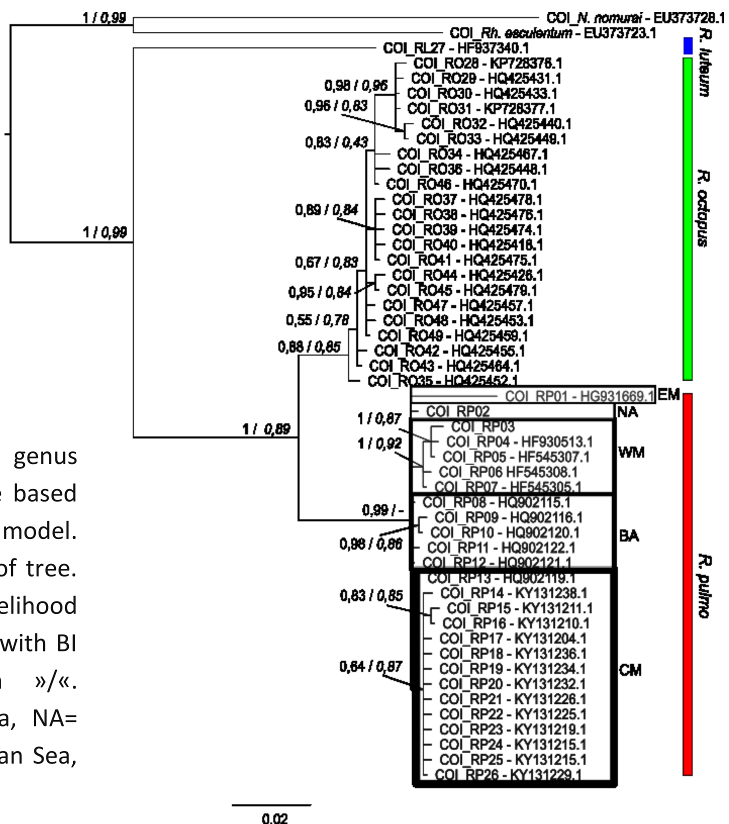

Figure 1: Phylogenetic relationships within genus Rhizostoma calculated by Bayesian inference based on COI sequences under a GTR+I evolution model. Numbers at nodes indicate relative support of tree. Relative support calculated with Maximum likelihood method is added in italics. Values calculated with BI and ML method are separated with »/«. Abbreviations: EM= East Mediterranean Sea, NA= North Adriatic Sea, WM = West Mediterranean Sea, CM= Central Mediterranean Sea.

Phylogenetic signal for species delimitation from nuclear DNA (28S, ITS1, ITS2) was less significant than from the mitochondrial DNA (COI). The phylogenetic COI tree revealed separation of the genus *Rhizostoma* into three species which is well supported. We proposed that species *R. pulmo* and *R. luteum* are the result of allopatric speciation considering their geographic separation and reproductive isolation. Relationships between *R. octopus* and *R. luteum* were not clarified in such details, since there was a lack of individuals from neighbouring locations. The Strait of Gibraltar was in the past an important biogeographic barrier, which prevented gene flow among populations of many species, but this is not the simple rule (Patarnello et al., 2007 and references therein). Nowadays, the most likely reason for continuous separation of *R. pulmo* and *R. luteum* is the Almeria – Oran front. This significant barrier is generated by inflow of less dense water from the Atlantic Ocean to the Mediterranean Sea, moreover this gradient creates two anticyclone circulations at Alboran Sea and prevents mixing of water with the rest of the Mediterranean Sea towards central and eastern parts (Tintore et al., 1988). The separation of the genus into three species is the result of biogeographic events in the past and ocean currents that still maintain this separation between the western part of the Mediterranean Sea and the central part.

Haplotype network analysis

We analysed 140 individuals and found 29 COI haplotypes, 14 private haplotypes which were found at just one geographic region. One haplotype (RP_COI_Hap_13) was shared between North East Atlantic and West Mediterranean. Star-like arrangement around haplotype number 23 is visible, which can be result of recent expansions from one smaller population. We found 9 ITS1 haplotypes from 50 individuals among which one haplotype (RP_ITS1_Hap_6) was shared between North Adriatic Sea, West, East and Central Mediterranean Sea. Analyses of 60 individuals of ITS2 showed 11 haplotypes. Haplotype RP_ITS2_Hap9 was shared between individuals from North Adriatic Sea, Central and West Mediterranean Sea. Moderate population structure of *R. pulmo* and *R. octopus* is visible. Differences between populations from East (EM), Central (CM) and West (WM) Mediterranean and North Atlantic Sea (NA) are present and can be visible on tree as four groups (Fig. 1) as well on haplotype network (Fig. 2).

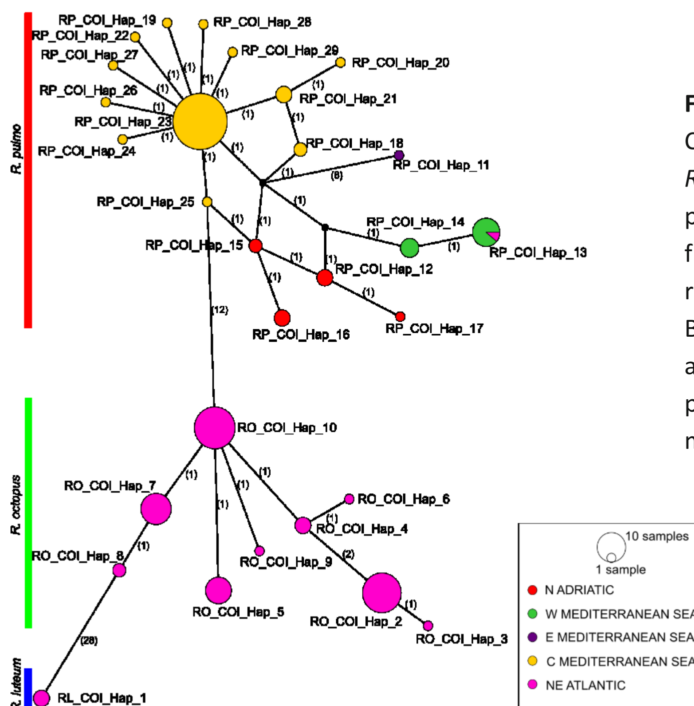

Figure 2: Median-joining network of COI haplotypes from genus *Rhizostoma*. The size of circles is proportional to the haplotype frequency. The colour of circles represents location of individuals. Black circles represent hypothetical ancestral haplotypes. Numbers in parenthesis represent number of mutations.

The strait of Messina is separates the east and west Mediterranean Sea, which can be the reason for the genetic differences among populations. Also, currents, temperature and salinity can create biogeographic regions and can hinder gene flow which is then detected as genetic differences among populations. Furthermore, cold and warm geological periods in the past caused significant fragmentation of habitats which decreased gene flow among populations (Faleh et al., 2017). There was also visible population structure among individuals of *R. octopus* from Ireland, Great Britain and France. Gene flow can be affected by currents, genetic barrier and different year of sampling. (Lee et al., 2013).Our results showed shallow geographic structure within *R. pulmo* and *R. octopus* which could be the result of currents, past geography and biology of this species with metagenetic life cycle. In the future, the population structure of *Rhizostoma* species can be solved in more detail by the use of genetic markers with fast mutation rates such as microsatellites.

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SL9

ARE DIFFERENT Escherichia coli POPULATIONS REFLECTED IN METAGENOMIC DATA?

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POVZETEK

Z razvojem tehnik novih generacij sekvenciranja (NGS) je raziskovanje mikrobiote doživelo pravi razcvet. Vendar pregled rezultatov NGS iz literature pogosto kaže, da zaradi naravno raznolike mikrobiote ne vidimo jasnih korelacij med sestavo mikrobiote in preučevanim fenotipom gostitelja. Poleg tega zaradi plastičnosti genomov nekaterih bakterijskih vrst lahko na fenotip gostitelja vplivajo le določeni sevi , ki jih z NGS ne razlikujemo med seboj. Zato smo v naši raziskavi vzporedno z NGS genotipizirali izolate bakterije *Escherichia coli* (*E. coli*), ki ne sodi med številčno najbolj zastopane mikroorganizme črevesne mikrobiote, a je lahko pomemben kazalec določenih dogajanj v gostitelju . Bakterija *E. coli* lahko s horizontalnimi prenosi pridobi genske zapise, povezane s fitnesom in virulenco. Ti geni so odgovorni za zmožnost prilagajanja ter za povzročitev bolezni. Genotipizirali smo 137 ne-klonalnih izolatov *E. coli*, pridobljenih iz fecesa 46 nosečnic z gestacijskim diabetesom. Vzporedno smo iz fecesa izolirali celokupno DNA in naredili 16S meta-taksonomsko analizo. Rezultate smo korelirali s podatki o prehranskih in drugih navadah nosečnic, življenjskem okolju ter osnovnih serumskih biokemijskih parametrih. Ugotovili smo zanimive povezave, npr. višja β-diverziteta pri nosečnicah, kjer smo uspeli izolirati več kot 1 ne-klonalni sev *E. coli* v primerjavi z nosečnicami, kjer smo izolirali zgolj 1, ter tudi korelacije, npr. med uživanjem pripravkov železa ter prisotnostjo sideroforjev in filogenetsko skupino *E. coli*.

Ključne besede: *Escherichia coli*, genotipizacija, metagenomski podatki

ABSTRACT

With the advent of Next Generation Sequencing (NGS) techniques, microbiota exploration has flourished. However, we often find that NGS data do not always show clear correlations between microbiota composition and the studied host phenotype, due to microbiota interindividual diversity. In addition, due to genome plasticity of certain bacterial species, only particular strains that cannot be distinguished by NGS, can affect the host phenotype. Therefore, in addition to NGS, we genotyped isolates of *Escherichia coli* (*E. coli*), which is not among the most abundant microorganisms in the gut microbiota but may be an important indicator of certain events in the host. Horizontal gene transfer (HGT) may increase *E. coli* fitness and virulence. The genes acquired by HGT may be responsible for the ability to adapt and cause disease. We genotyped 137 non-clonal *E. coli* isolates obtained from the faeces of 46 pregnant women with gestational diabetes. In parallel, DNA was isolated from the faeces and a 16S meta-taxonomic analysis was performed. The results were correlated with lifestyle characteristics, living environment and basic serum biochemical parameters. We found interesting associations, e. g. higher β-diversity in pregnant women with more than 1 non-clonal strain of *E. coli*

compared to pregnant women with only 1, as well as correlations, e. g. between the consumption of iron preparations and the presence of siderophores and the phylogenetic group of *E. coli*.

Key words: *Escherichia coli*, genotyping, metagenomic data

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SL10

GENETIC DIVERSITY OF DREŽNICA GOAT

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POVZETEK

Genomske analize pri rejnih živalih so poleg očitnega doprinosa k napredku živinoreje, razkrile pomembna spoznanja z vidika ohranjanja pestrosti vrst. Drežniška koza (*Capra aegagrus hircus*) je edina slovenska avtohtona pasma koz in ima status kritično ogrožene pasme. Njena odpornost na bolezni in prilagojenost na skromne razmere alpskega okolja so ključni pokazatelji, da predstavlja pomemben živalski genski vir, ki ga je potrebno ohraniti. Da bi preverili genetsko strukturo pasme in njen izvor znotraj populacije koz, smo genotipizirali 135 drežniških koz z Illumina čipom s ~ 50.000 SNP označevalci. V prvem sklopu smo z bioinformacijskimi orodji analizirali parametre genetske raznolikosti znotraj pasme ter genetsko sorodnost s pasmami iz alpskega okolja. Drežniške koze si na visokogorskih pašnikih delijo habitat z alpskim kozorogom (*Capra ibex*), zato smo genotipizirali tudi devet kozorogov in potencialnega križanca med kozo in kozorogom. V drugem sklopu smo preverili, ali gre za dejanskega križanca med omenjenima vrstama, kar nakazuje njegov fenotip. Rezultati analiz iz prvega sklopa so pokazali, da ima pasma veliko število privatnih alelov. Z ocenami genetskih razdalj smo dokazali, da ima drežniška koza neodvisen izvor in izjemno majhen delež primesi drugih pasem. Z analizo v drugem sklopu smo potrdili, da gre za dejanskega križanca med samcem kozoroga in samico drežniške koze, kar pomeni, da med obema vrstama lahko pride do parjenja in da imajo takšni križanci možnost preživetja. Raziskava je pomembna z vidika temeljnih in evolucijskih genetskih raziskav pri ohranjanju kritično ogroženih pasem in pri upravljanju z divjimi sorodnimi vrstami.

Ključne besede: drežniška koza, SNP genotipizacija, genetska raznolikost, alpski kozorog

ABSTRACT

Genomic analyses in farm animals have beside the obvious contribution to livestock development, revealed insights into species diversity important for conservation programs. Drežnica goat (*Capra aegagrus hircus*) is the only Slovenian indigenous goat breed with critically endangered status above all. Its disease resistance and adaptability to the modest conditions of Alpine environment are important traits worth conserving. To analyse the genetic structure of the breed and its origin within goat population, 135 Drežnica goats were genotyped with an Illumina chip containing ~ 50.000 SNP genetic markers. In the first part, we analysed genetic diversity parameters within the breed and genetic relationship to other breeds from Alpine area using bioinformatics tools. Drežnica goat share habitat with Alpine ibex (*Capra ibex*) in mountain pastures, therefore we genotyped nine ibexes and a presumed hybrid between goat and ibex. In the second part, we checked whether this animal is an actual hybrid as was suggested by its phenotype. The results from the first part showed that the breed had a large number of private alleles. Estimated genetic distances demonstrated that Drežnica goat has distinct genetic origin and an extremely low introgression of other breeds. In the second part of analysis confirmed that the hybrid is a result of a male ibex and a female goat crossing, meaning that such matings can occur between these two

species and those hybrids have a potential to survive. Ongoing research is important for basic and evolutionary genetic studies in conservation of critically endangered breeds as well as for the management of wild related species.

Key words: Drežnica goat, SNP genotypization, genetic diversity, Alpine ibex

MARKING THE 50th ANNIVERSARY OF GENETIC SOCIETY OF SLOVENIA

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GENETIC RESEARCH IN SLOVENIA 1920–1990

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POVZETEK

V Sloveniji je genetske raziskave začel Fran Jesenko (1875-1932). Predno je postal leta 1920 profesor botanike na Univerzi v Ljubljani je bil učitelj na univerzah na Dunaju in v Zagrebu. Znan je bil predvsem po raziskavah fertilnosti križancev pšenice in rži, v uglednih publikacijah tedanjega časa je objavil odmevna dela v nemščini, francoščini in angleščini. Znan je postal predvsem po predavanju o fertilnosti medvrstnih križancev pri rastlinah na 4. Mednarodni konferenci genetike v Parizu leta 1911. V sodelovanju s Francem Mikužem je izvajal poskuse na poskusnem polju v bližini Beltinec v Prekmurju. Mikuž je tam raziskoval in žlahtnil pšenico in ajdo ter je bil v času med obema vojnama zelo aktiven član Eucarpije, evropskega združenja za raziskave žlahtnjenja rastlin. Tudi Slovenci, učitelji in raziskovalci na področju rastlinske genetike na Univerzi v Zagrebu Alojz Tavčar, Marija Kump, Svetka Jamšek Korić in Marijan Jošt so bili aktivno strokovno povezani s Slovenijo. Na področju humane genetike je svoje raziskave v tem času začel profesor Božo Škerlj. Leta 1969 je bilo med potekom kongresa biologov Jugoslavije ustanovljeno Društvo genetikov Slovenije, prvotno povezano v zvezo društev genetikov Jugoslavije, ki pa je kmalu postalo povsem samostojno.

Ključne besede: genetika, zgodovina, društva, Slovenija

ABSTRACT

Genetic research in Slovenia was started by Fran Jesenko (1875-1932). In 1920 Jesenko, former teacher at Universities in Vienna and Zagreb, was appointed as professor of botany at Ljubljana University. His research on fertile wheat-rye interspecific hybrids was published in important international scientific publications of the time, in German, French and English. He was well known especially by his presentation on fertility problems in interspecific hybrids, on the 4th International Conference on Genetics in Paris in 1911. F. Jesenko cooperated with Franc Mikuž, head of experimental and plant breeding station near Beltinci in Prekmurje. Mikuž was in Prekmurje breeding wheat and buckwheat, and was known in the period prior to 1941 as an active member of Eucarpia, European association for research in plant breeding. Slovenians Alojz Tavčar, Marija Kump, Svetka Jamšek Korić and Marijan Jošt, involved in Zagreb plant genetics activities, had some impact on the development of the field in Slovenia too. Human genetics was in the same time studied in Ljubljana by professor Božo Škerlj. In 1969, during the sessions of the Biology Congress of Yugoslavia

in Ljubljana, it was established Society of Geneticists of Slovenia, in the beginning connected with Yugoslav Genetic Association, but it soon became independent.

Key words: genetics, history, societies, Slovenia

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GENETIC SOCIETY OF SLOVENIA IN THE PERIOD FROM 1977 TO 2019

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POVZETEK

Slovensko genetsko društvo (SGD) je bilo ustanovljeno leta 1992 kot naslednik Društva slovenskih genetikov, ki je bilo eno od nacionalnih društev genetikov, ki je delovalo pod okriljem Zveze jugoslovanskih genetskih društev, ki je bila ustanovljena leta 1977. Jugoslovansko Genetsko društvo, ki je bilo njen predhodnik, je bilo ustanovljeno leta 1969 v Ljubljani in je začelo z izdajanjem revije Genetika, ki je bila takrat pomembna komunikacijska vez med jugoslovanskimi genetiki. Po prvih dveh kongresih v Dubrovniku (1976) in Vrnjački Banji (1981) je Društvo slovenskih genetikov leta 1987 v Ljubljani organiziralo tretji kongres jugoslovanskih genetikov. Leta 1992 se je Društvo slovenskih genetikov reorganiziralo v Slovensko genetsko društvo (SGD) z namenom promocije in razvoja genetike na različnih področjih znanosti. Pomemben cilj društva so predstavljali poskusi prispevati k bolj znanstveno utemeljenim javnim razpravam o sodobnih genetskih raziskavah. SGD je povezalo raziskovalce na področju humane, živalske, rastlinske in mikrobne genetike, ki so v svojih raziskavah uporabljali klasični, kvantitativni in molekularni pristop. Člani SGD zastopajo različne poklice, vključno z zdravniki, biologi, mikrobiologi, biokemiki, živinorejci in žlahtnitelji rastlin, genetiki, biotehnologi in bioinformatiki. Humani genetiki so bili v prvih letih vodilna struja v SGD, kar se je odrazilo tudi v ustanovitvi posebnega odseka za humano genetiko znotraj društva. Številne sorazmerno mlade skupine so postale zelo aktivne na področju genetike in so svoje delo promovirale z različnimi mednarodnimi publikacijami in na mednarodnih srečanji. V tem prvem obdobju SGD so člani SGD, Nina Canki, Franc Ločniškar, Jelisava Adamič, Miklavž Grabnar in Peter Lazar, pomembno prispevali k hitremu razvoju genetskih raziskav v Sloveniji. Prvi kongres SGD z mednarodno udeležbo je bil v Ljubljani leta 1997 in od takrat so se kongresi redno organizirali vsaka tri leta. Leta 2009 je bil organiziran skupni kongres SGD in Slovenskega biokemijskega društva z namenom spodbujanja sodelovanja med člani obeh društev. Trenutno je SGD pomemben partner v javnih razpravah, pri pripravi zakonodaje na področju genetskih raziskav in uporabe gensko spremenjenih organizmov ter pri izobraževanju in oblikovanju javnega mnenja o novih pristopih v genetiki in genomiki.

Ključne besede: genetika, raziskave, društvo, Slovenija

ABSTRACT

The Genetics Society of Slovenia (GSS) was established in 1992 as a successor of the Society of Slovenian Geneticists which was one of the national societies of geneticists, which were organised under the auspices of the Union of the Yugoslav Genetic Societies in 1977. The Yugoslav Genetic Society was established in 1969 in Ljubljana and published the journal Genetika which was an important communication link for Yugoslav geneticists at that time. After the first two congresses which were held in Dubrovnik (1976) and Vrnjacka Banja (1981), the Society of Slovenian Geneticists organised the third Congress of Yugoslav Geneticists in 1987 in Ljubljana. In 1992, the Society of

Slovenian Geneticists was reorganised into Genetics Society of Slovenia (GSS) with the aim to promote and develop genetics in various fields of science. An important goal of the society were also the attempts to contribute to more scientifically based public debates on contemporary genetic research. GSS joined researchers in the field of human, animal, plant and microbial genetics implementing classical, quantitative and molecular approach in their research. The members of GSS come from different professions including physicians, biologists, microbiologists, biochemists, animal and plant breeders, geneticists, biotechnologists and bioinformaticians. In the first few years, the human geneticists were the flagship of the GSS, and this was reflected also in establishing of an own section for human genetics within the society. A number of relatively young groups became very active in the field of genetics and got their work promoted through various international publications and meetings. In this first period of GSS, Nina Canki, FrancLočniškar, Jelisava Adamič, Miklavž Grabnar and Peter Lazar were important members of GSS who contributed significantly to the rapid development of genetic research in Slovenia. The first congress of GSS with international participation was held in Ljubljana in 1997 and since then congresses were organized on the regular basis every three years. In 2009 the joint congress of GSS and Slovenian Biochemical Society was organized with the aim to promote collaboration between members of both societies. At present, GSS is an important partner in public discussions, preparation of legislative in the field of genetic research and use of genetically modified organisms as well as education and creation of public opinion on novel approaches in genetics and genomics.

Key words: genetics, research, societies, Slovenia

POSTERS

POSTERS – MOLECULAR BASIS OF DISEASES

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P1

IDENTIFICATION OF THE CANDIADTE REGION FOR MASTITIS RESISTANCE IN HOLSTEIN FRISIAN DAIRY COWS

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POVZETEK

Visoko mlečnost pri kravah molznicah pogosto spremlja manjša odpornost na mastitis, kar povzroča znatne izgube v mlečni industriji. Da bi zmanjšali pojavnost subkliničnega in kliničnega mastitisa, izboljšali dobrobit živali in znižali stroške za veterinarske posege, se mnogi rejski programi osredotočajo na odkrivanje kandidatnih genov, povezanih z odpornostjo na mastitis. Na odpornost na mastitis vpliva več genov, ki so vpleteni v različne biološke poti. V trenutni študiji smo izvedli rudarjenje po literaturi in javno dostopnih podatkovnih zbirkah, da smo identificirali kandidatne gene in kvantitativne lokuse (*angl., quantitative trait loci, QTL*), povezane z dovzetnostjo za mastitis. Z bioinformacijsko analizo smo identificirali gen, ki se nahaja na BTA5, protein tirozin fosfatazni receptor tipa R (*PTPRR*), ki je bil že prej povezan z lastnostmi mlečnosti pri govedu (Tolleson in sod., 2017) in se nahaja v neposredni bližini QTL-ov za količino mleka in število somatskih celic (Jiang in sod., 2019). Pri miši je bila družina genov PTP povezana z involucijo in remodeliranjem mlečne žleze (Aoki in sod., 1999). Poleg tega je bilo opaženo inhibitorno delovanje genov PTP na gene, močno izražene med laktacijo. Regulatorno vlogo PTP so potrdili tudi pri razvoju mlečne žleze, medtem ko se njihovo izražanje v fazi involucije po odstavitvi vrne skoraj na osnovno raven. Izkaže se, da imajo PTP ključno vlogo pri uravnavanju temeljnih celičnih procesov, kot so proliferacija, diferenciacija in razvoj. Točkovna mutacija rs29014396 v regiji znotraj gena *PTPRR* pri govedu je bila izbrana na osnovi prioritizacije genetskih variant. V bližini tega polimorfnega mesta se nahaja miR2284Z-5, ki je povezana z lastnostmi mlečnosti (Strillacci in sod., 2014) in diferenciacijo celic. Izvedli smo test PCR-RFLP za to polimorfno mesto na vzorcu črno - belega goveda, kjer smo imeli dve skupini, živali dovzetne in odporne na mastitis. Rezultati genotipizacije kažejo značilno razliko v frekvencah alelov med obema skupinama. V prihodnjih raziskavah se bomo osredotočili na mutacije v izbrani kandidatni regiji pri drugih pasmah in na povezave s proizvodnimi ter z zdravjem povezanimi lastnostmi.

Ključne besede: *Bos taurus*, imunski odziv, *miR2284Z*-5, mastitis, *PTPRR*

ABSTRACT

High level of milk production in dairy cows is often accompanied with lower resistance to mastitis, which causes significant losses in dairy industry. In order to reduce the incidence of subclinical and clinical mastitis, to improve animal welfare and to reduce costs for veterinary interventions, many breeding programmes focus on detection of candidate genes associated with mastitis resistance. However, mastitis resistance is influenced by several genes that are involved in different biological pathways. In the current study, we performed literature and data mining to identify candidate genes and quantitative trait loci (QTL) associated with susceptibility to mastitis. Bioinformatics analysis revealed the gene positioned at BTA5, protein tyrosine phosphatase receptor

type R (*PTPRR)*, which has previously been associated with udder traits in cattle (Tolleson et al., 2017) and is in close proximity to QTL for milk yield and somatic cell score (Jiang et al, 2019). In mouse, the possible contribution of the members of the PTP gene family to the development, involution, and remodelling of the mammary gland has been suggested (Aoki et al., 1999). In addition, their possible inhibitory action on high expression of milk genes during lactation has been proposed. The regulatory role of PTPs was found in the development of the mammary gland, whereas their expression is returned almost to the virgin level at the involution stage after weaning. PTPs have been shown to play critical role in regulating fundamental cellular processes such as proliferation, differentiation and development. Point mutation rs29014396 within the region of the bovine *PTPRR* gene was selected after the variant prioritization step of our analysis. Close to this polymorphic site *miR2284Z-5* is located, which is also associated with milk traits (Strillacci et al., 2014) and cell differentiation. Moreover, we performed PCR-RFLP test for this polymorphic site in our sample of Holstein-Friesian cows, susceptible and resistant to mastitis. The results of genotyping show significant difference in allele frequencies between the mastitis and healthy groups. Our future research will be focused on possible associated mutations in this region in other breeds and on associations with health and production traits.

Key words: *Bos taurus*, immune response, *miR2284Z*-5, mastitis, *PTPRR*

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P2

CONTRIBUTION OF *Rpi-Smira2/R8* GENE TO LATE BLIGHT RESISTANCE IN POTATO R8 PLANTS

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POVZETEK

Krompirjeva plesen (*Phytophthora infestans*) ogroža pridelek krompirja po celotnem svetu. Skupaj s sodobnimi tehnikami žlahtnjenja in piramidenja odpornostnih (*R*) genov so bile razvite nove sorte z visoko in trajno odpornostjo. Ena izmed njih je tudi Sarpo Mira s petimi glavnimi geni *R* (*R3a*, *R3b*, *R4*, *Rpi-Smira1*, *Rpi-Smira2/R8*). Nedavne raziskave na tej sorti so pokazale visoko odpornost na nivoju cele rastline, medtem ko so bili posamezni odrezani listi (DLA) občutljivi. Cilj raziskave je bil razviti rastline z genom *R8* in preveriti njihovo odpornost. Celotno potomstvo petih križanj med Sarpo Miro in občutljivimi sortami je bilo preverjeno z genskimi markerji za prisotnost genov *R3a*, *R3b*, *Rpi-Smira1* in *R8*. Za preverjanje prisotnosti gena *R4* smo uporabili agroinfiltracijo z *Agrobacterium tumefaciens*, ki je v svojem plazmidu vseboval gen za efektor Avr4. R8-rastline bomo izpostavili različno agresivnim izolatom *P. infestans* pod različnimi okoljskimi pogoji (DLA, rastlinjak, poljski poskus). Stopnjo okuženosti krompirjeve plesni bomo določili vizualno glede na delež prisotnih listnih nekroz na podlagi katerih bomo za vsako rastlino določili stopnjo odpornosti. Hkrati bomo spremljali tudi izražanje gena *R8* ter drugih genov povezanih z odzivom na biotski stres. Na podlagi pridobljenih rezultatov bomo lahko določili nivo odpornosti rastlin in s tem doprinos gena *R8* k visoki odpornosti sorte Sarpo Mira.

Ključne besede: *Phytophthora infestans*, Sarpo Mira, gen *R8*, izražanje genov

ABSTRACT

Late blight (*Phytophthora infestans*) is a significant threat to potato yields across the globe. Improved breeding techniques in combination with resistance (*R*) gene pyramiding resulted in varieties with a high level of resistance. One of them is Sarpo Mira with five major *R* genes (*R3a*, *R3b*, *R4*, *Rpi-Smira1*, *Rpi-Smira2/R8*). Several observations of contradicting resistance have been observed for this variety between whole plant level and detached leaf level (DLA). Our goal was to develop plants containing solely *R8* gene and observe their resistance to late blight. Progenies of five crosses between Sarpo Mira and susceptible varieties were screened with gene markers for *R3a*, *R3b*, *Rpi-Smira1*, and *R8* gene. For *R4* gene screening agroinfiltration with *Agrobacterium tumefaciens* containing *Avr4* effector was applied. For resistance testing, R8 plants will be subjected to several environmental conditions (DLA, whole plant in greenhouse and field trial) and a number of *P. infestans* isolates with various aggressiveness. Late blight severity will be assessed visually as estimated percent coverage of foliar lesions, determining a resistance level for each plant. Simultaneously gene expression of *R8* gene and other biotic stress response genes will be observed. Combination of all listed factors and conditions will enable us to determine the level of resistance to late blight in R8 plants and impact of *R8* gene in high resistance in Sarpo Mira.

Key words: *Phytophthora infestans*, Sarpo Mira, *R8* gene, gene expression

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P3

A CHOLECYSTOKININ A RECEPTOR POLYMORPHISM rs6448456 AFFECTS LIPID LEVELS AND RESPONSE TO GLUCOSE IN PATIENTS WITH POLYCYSTIC OVARY SYNDROME

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POVZETEK

Sindrom policističnih ovarijev (PCOS) je ena najpogostejših endokrinih in presnovnih motenj pri ženskah v rodni dobi. Za zdravljenje presnovnih motenj veliko obeta razvoj agonistov receptorjev za holecistokinin (CCK), saj CCK poveča izločanje inzulina, poveča rast trebušne slinavke in zmanjša občutek lakote. Deluje prek vezave na receptorja za holecistokinin A (CCKAR) in B (CCKBR). Ker so v genih *CCKAR*-ja in *CCKBR*-ja pogosti številni funkcionalni polimorfizmi, bi slednji lahko pomembno vplivali na uspešnost zdravljenja. Prav tako bi lahko povzročili zmanjšano ali okrepljeno delovanje endogenega CCK. V raziskavi smo preverjali vpliv polimorfizmov *CCKAR*-ja in *CCKBR*-ja na antropometrične in presnovne značilnosti bolnic s PCOS-om. Vključili smo 168 bolnic. Izvedli smo genotipizacijo za dva polimorfizma v genu *CCKAR* in za štiri v genu *CCKBR*. Pri statistični analizi smo uporabili dominantni model. Nosilke polimorfizma CCKAR-ja rs6448456 so imele statistično značilno višjo koncentracijo celokupnega holesterola in LDL-ja ter značilno nižje koncentracije glukoze v krvi po 30, 60 in 90 minutah obremenilnega testa z glukozo. Drugih pomembnih, statistično značilnih vplivov polimorfizmov *CCKAR*-ja in *CCKBR*-ja na značilnosti bolnic s PCOS-om nismo zaznali. Naši rezultati nakazujejo vpliv polimorfizmov *CCKAR*-ja na presnovne značilnosti bolnic s PCOS-om, a ker je bila naša raziskava prva tovrstna, so potrebne nadaljnje raziskave, ki bodo ovrednotile naše izsledke.

Ključne besede: sindrom policističnih ovarijev, holecistokinin, holecistokininski receptor A, holecistokininski receptor B

ABSTRACT

Polycystic ovary syndrome (PCOS) is one of the most common endocrine and metabolic disorders in women of the reproductive age. It is expected that cholecystokinin (CCK) receptor agonists might improve treatment of the metabolic disorders due to CCK's incretin effect, regulation of pancreas growth and mediation of satiety. Biological actions of CCK are mediated by cholecystokinin receptor A (CCKAR) and B (CCKBR). As many common functional polymorphisms are present in both genes, they could have a significant impact on the treatment. Furthermore, they could lead to a reduced or enhanced function of endogenous CCK. We have investigated the influence of *CCKAR* and *CCKBR* polymorphisms on the anthropometric and metabolic characteristics of patients with PCOS. We enrolled 168 patients. Genotyping was performed for two polymorphisms in the *CCKAR* gene and for four in the *CCKBR*. The carriers of the *CCKAR* rs6448456 polymorphism had significantly higher concentrations of total cholesterol and LDL, and significantly lower blood glucose concentrations after 30, 60 and 90 minutes at oral glucose tolerance test. No other relevant and significant associations between polymorphisms and characteristics of patients with PCOS were detected. Our

results suggest that *CCKAR* polymorphisms influence metabolic characteristics of patients with PCOS. Since our research was the first of its kind, further studies are needed to validate our results.

Key words: polycystic ovary syndrome, cholecystokinin, cholecystokinin receptor A, cholecystokinin receptor B

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P4

DEVELOPMENT OF A SYSTEM FOR DNA-PROTEIN INTERACTION PARTNERS DISCOVERY

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POVZETEK

Interakcije proteinov z DNA usmerjajo številne celične procese, med katerimi je tudi natančno časovno uravnavanje prepisovanja. Odkrivanje regulatornih elementov DNA in transkripcijskih faktorjev ter razumevanje njihovih medsebojnih interakcij so izrednega pomena, saj lahko pojasnijo razvoj marsikatere bolezni in odpirajo nove možnosti za zdravljenje. Metode za odkrivanje interakcijskih partnerjev znane DNA omogočajo odkrivanje močnih in stabilnih interakcij, medtem ko šibkih in prehodnih pogosto ne moremo zaznati. Da bi omogočili odkrivanje vseh vrst interakcij, smo razvili nov pristop za odkrivanje interakcijskih partnerjev DNA na osnovi označevanja bližnjih molekul z biotinom. Temelji na uporabi trikomponentnega sistema, ki sestoji iz nukleotidnega zaporedja F-DNA in dveh fuzijskih proteinov, Tus-TurboID in *GAL4*(1–147)-sfGFP. F-DNA vsebuje preučevano zaporedje DNA in mesti za vezavo obeh fuzijskih proteinov, tako da so komponente sistema in iskani interakcijski partnerji kolokalizirani. Fuzijski protein Tus-TurboID vsebuje biotin ligazo, ki ob dodatku biotina z njim označi bližnje proteine, tj. interakcijske partnerje preučevane DNA in protein *GAL4*(1–147)-sfGFP, ki služi kot pokazatelj pravilnega delovanja sistema. Sistem je mogoče uporabiti tako *in vivo* kot *in vitro*. Za dokaz koncepta smo pripravili kontrolni *in vitro* sistem z znanim kompleksom DNA–protein, GADD–p53, katerega delovanje je potrebno nadaljnje optimizirati.

Ključne besede: interakcije DNA–protein, trikomponentni sistem, biotinilacija

ABSTRACT

DNA–protein interactions govern many cellular processes, including precise temporal regulation of transcription. Discovery of DNA regulatory elements and DNA-binding transcription factors, as well as understanding of their interactions, are of great importance for they provide insight into the development of many diseases, allowing the design of new treatments. Methods for detection of protein interaction partners of a known DNA are suitable for the detection of strong and stable interactions, however, weak and transient ones are often overlooked. To be able to detect all types of interactions, we set on to develop a novel approach for DNA–protein partners discovery, which is based on a three-component system that enables enzyme-catalysed proximity labeling of proteins with biotin. The system consists of a nucleotide sequence F-DNA and two chimeric proteins, Tus-TurboID and *GAL4*(1–147)-sfGFP. F-DNA contains DNA sequence of interest and binding sites for chimeric proteins, so the components of the system and target interaction partners are colocalised. Tus-TurboID contains a mutant biotin ligase which biotinylates colocalised proteins on F-DNA, whereas *GAL4*(1–147)-sfGFP represents the internal control of the system's functionality. The system can be used either *in vivo* or *in vitro*. As a proof-of-concept we prepared an *in vitro* system involving a known DNA–protein complex, GADD–p53. To be applicable, the system has to be further optimised.

Key words: DNA–protein interactions, three-component system, biotinylation

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P5

EXPRESSION ANALYSIS OF *LIF*, *JUNB*, *IL6*, *FOS*, *IL10* and *SOCS3* GENES IN EUTOPIC ENDOMETRIUM DURING WINDOW OF IMPLANTATION IN WOMEN WITH ADENOMYOSIS

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IZVLEČEK

Adenomiza je bolezen, kjer se endometriju podobno tkivo razrašča znotraj miometrija. Domnevno naj bi adenomioza vplivala na receptivnost endometrija za ugnezditev zarodka. Študije na celotnem genomu pri sorodni endometriozi, za katero je značilno razraščanje endometriju podobnega tkiva izven maternice, so v endometriju v času receptivnosti zaznale številne spremembe v bioloških mehanizmih. Namen naše študije je bil, glede na poznavanje endometrioze, izbrati in validirati kandidatne gene v eutopičnem endometriju v času okna implantacije pri adenomiozi. V ta namen smo na podlagi analize literature razvili kataloga s 207 in 32 genov, povezanih z endometriozo oz. adenomiozo. Sledila je obogatitvena analiza bioloških poti za vsako bolezen, kjer smo iz skupne poti »*signalizacija interleukina-4 in interleukina-13*« izbrali kandidatne gene: *LIF*, *JUNB*, *IL6*, *FOS*, *IL10* in *SOCS3*. V raziskavo so bili vključeni vzorci endometrija žensk z adenomiozo, endometriozo in kontrol. Validacija je pokazala trend spremenjenega izražanja *LIF* pri adenomiozni skupini v primerjavi s kontrolami, vendar podatki niso značilni. Bioinformacijska primerjava mrež in bioloških poti med dobro raziskano endometriozo in slabo raziskano adenomiozo omogočajo prepoznavanje novih kandidatnih genov s potencialno vlogo pri adenomiozi. V prihodnje bo potrebno analizo razširiti na večje število vzorcev.

Ključne besede: endometrijska receptivnost, adenomioza, pristop mrež in bioloških poti, validacija genov

ABSTRACT

Adenomyosis is a disease where tissue similar to endometrium grows within myometrium. It was suggested that adenomyosis affects endometrial receptivity for embryo implantation. However, many dysregulated mechanisms in eutopic endometrium during window of implantation (WOI) have been detected by genome-wide profiling in endometriosis, which is characterized by presence of ectopic endometrium outside of the uterus. The aim of our study was, based on knowledge regarding endometriosis, select and validated gene candidates in eutopic endometrium during WOI in adenomyosis. Through literature screening we therefore, developed catalogues with 207 and 32 genes, associated with endometriosis and adenomyosis, respectively. Enrichment pathway analysis followed for both gene lists and from common "*Interleukin-4 and Interleukin-13 signalling*" pathway gene candidates were selected: *LIF*, *JUNB*, *IL6*, *FOS*, *IL10*, and *SOCS3*. Samples of endometrium were collected from adenomyosis, endometriosis cases and controls. Despite no significant differences, the trend of alter expression levels in *LIF* was observed in adenomyosis compared to controls. Bioinformatics comparison of pathways and networks between well studied endometriosis and

poorly studied adenomyosis may suggest new gene candidates with a potential role in adenomyosis. Experimental validation is needed in larger number of samples.

Key words: endometrial receptivity, adenomyosis, pathway and network-based approach, gene validation

INTRODUCTION

Establishment of pregnancy requires interaction between vital embryo and receptive endometrium to facilitate apposition, attachment and invasion of the blastocyst and migration of endometrial stromal cells toward the site of implantation causing blastocyst-induced tissue remodelling (1). Scoring system for optimal embryo selection in assisted reproductive technology (ART) protocols is well developed (2), but not for endometrium parameters (3). Endometrium, an inner epithelial layer of the uterus, responds to periodically fluctuations of ovarian sex hormones and becomes receptive for embryo implantation in mid-secretory phase of the menstrual cycle known as WOI (4).

Advanced imaging technology, such is transvaginal ultrasound (TVUS), now enables detect uterine pathology such as adenomyosis that was previously possible diagnosed after hysterectomy (5). Adenomyosis is characterized as the presence of tissue similar to endometrium within the smooth muscle of the uterus called myometrium. Its prevalence vary among studies due to no exact criteria for sonographic features of adenomyosis (6). Limited number of studies indicate that adenomyosis interference with endometrial receptivity, but molecular background of mechanisms is quite unknown (7). On the other hand, genome-wide approach has been widely adopted in related disease called endometriosis which is characterized by the presence of tissue similar to endometrium in pelvic cavity. Despite low overlap of high-throughput results, enriched pathways such as JNK/MAPK, PI3K-Akt and p53 signalling, adhesion junction (8), steroid hormone response (9) immune dysfunction and inflammatory response (10) have been suggested as possible dysregulated mechanisms in eutopic endometrium during WOI in women with endometriosis.

Due to low numbers of genome-wide studies performed in adenomyosis and pathophenotype similarity with endometriosis, we hypothesized that dysregulated pathways in eutopic endometrium during WOI in endometriosis may be dysregulated in eutopic endometrium during WOI in adenomyosis as well. Since biological knowledge accumulated in public databases (11), a pathway network-based approach may provide new meaningful grouping of genes, thus providing new dysregulated pathways characterizing observed phenotype (12). The first aim of our study was therefore to perform integrative analysis from published data to generate two networks of pathways characterizing eutopic endometrium during WOI in adenomyosis and endometriosis, respectively. The second aim was to select gene candidates from intersected pathway between both networks and validate their expression levels in samples of eutopic endometrium during WOI from adenomyosis, endometriosis cases and controls.

MATERIAL AND METHODS

Bioinformatics analysis: Literature screening was conducted in PubMed using keywords “adenomyosis” or “endometriosis” with combination: “eutopic endometrium”, “gene expression”, “transcriptomic”, “proteomic”, “epigenomic”, and “window of implantation”. For development of gene catalogue specific for adenomyosis, candidate-gene and genome-wide studies that analysed eutopic endometrium between women with and without adenomyosis during mid-secretory phase

of the menstrual cycle were retrieved. For development of gene catalogue specific for endometriosis, only genome-wide studies that compared eutopic endometrium between women with and without endometriosis during mid-secretory phase were retrieved. Further, top 15 up and/or down regulated transcripts/proteins were extracted. Gene nomenclature of extracted loci in adenomyosis and endometriosis cases has been edited according to HUGO Gene Nomenclature Committee (HGNC) database (13). Both gene catalogues were separately uploaded in Reactome database to perform enrichment pathway analyses. Among top 10 pathways from each disease, annotated genes from common pathway have been prioritized for expression validation by qPCR. Network interaction of gene candidates was visualised by STRING tool (14).

Participants. The study was approved by the National Ethic Committee of Republic of Slovenia (0120-259/2018/16) and written informed consent was obtained from all participants. Adenomyosis and endometriosis have been diagnosed by TVUS examination (General Electric Voluson E8). Sonographic criteria for adenomyosis diagnosis were: asymmetrical myometrial thickening not caused by the presence of fibroids, parallel shadowing, linear striations, myometrial cysts, hyperechoic islands, the presence of adenomyoma. Endometriosis was diagnosed when characteristic cysts of ground-glass appearance in the ovaries were seen or if there were subjective signs of deep infiltrating endometriosis seen as assessed by the expert sonographer. These would include thickening of the uterosacral ligaments, rectovaginal septum, the presence of endometriotic nodules in the pouch of Douglas, rectovaginal septum, bowel, urinary bladder or ureters. Women included in control group were free of adenomyosis, myoma, endometriosis and polycystic ovary syndrome (PCOS).

Sample collection and processing. Eutopic endometrium samples were collected from endometriosis (n = 4), adenomyosis (n = 7) and control (n = 8) groups using endometrial curette (Probet, Gynetics) on 7th day from luteinizing hormone (LH) surge (LH+7) determined by urinary LH ovulation rapid test cassette (AllTest). Endometrial biopsy was immediately placed in RNA later (Invitrogen) and stored overnight in +4°C, than stored in -20°C and within the same or next day transferred and stored in -80°C. Total RNA was extracted using the miRNeasy Mini Kit (Qiagen) standard protocol. Isolated RNA concentrations were measured spectrophotometrically (BioTek).

Validation by qPCR. Used volume of total RNA was calculated for final synthesis of 1 µg cDNA using SuperScript IV VILO Master Mix (Catalog number: 11756050, Invitrogen) according to the manufacturer's instructions. Selected mRNA with TaqMan gene expression assay (Applied Biosystems) IDs are: *LIF* (Hs01055668_m1), *JUNB* (Hs00357891_s1), *IL6* (Hs00174131_m1), *FOS* (Hs99999140_m1), *IL10* (Hs00961622_m1), and *SOCS3* (Hs01000485_g1). In total volume of 10 µL, each reaction contained 5 µL of TaqMan Gene Expression Master Mix (2X), (Catalog number: 4369016, Applied Biosystems), 0.5 µl of assay (20X), 2.5 µl of nuclease-free water and 2 µl of 10-times diluted cDNA template. Nuclease-free water and no reverse transcriptase cDNA were used as negative controls. Quantification was performed in a LightCycler480 instrument (Roche Applied Science). The conditions for qPCR were as follows: UNG incubation (50°C for 2min), polymerase activation (95°C for 10min), and 45 cycles of denature (95°C for 15s) and anneal/extend (60°C for 1min) steps. Each sample was performed in duplicates and average CT values were further used for calculations. Target *GAPDH* (Hs02786624_g1, Applied Biosystems) was used as the reference gene. Gene expression values were calculated based on the equation: $RQ = 2^{-\Delta\Delta CT}$ (15). The values of ΔCT were calculated as follows: *ΔCT (sample) = CT (sample) – CT (GAPDH)*. Normalized calibrator was calculated as an average of CT values of all samples for each investigated gene. The values ΔΔCT were calculated as follows: *ΔΔCT (sample) = ΔCT (sample) – CT (calibrator)*. Final results were expressed as fold

differences in gene expression relative to the normalized calibrator. Non-parametric Mann-Whitney U test was used for the statistical significance ($p<0.05$).

RESULTS AND DISCUSSION

Integrative analysis. Catalogue of genes, associated with endometriosis was developed from retrieved 7 studies. Among them, one was performed at epigenomic level with gene expression validation, five at transcriptomic and one at proteomic levels which altogether resulted in 207 genes. Gene catalogue, associated with adenomyosis was developed from 6 candidate-gene and one genome-wide studies, resulted in 32 genes. Gene catalogues for each disease are presented in Table 1. Enrichment pathway analyses resulted in 1148 and 358 pathways in endometriosis and adenomyosis, respectively. Top 10 pathways for each disease are presented in Table 2. Genes from both gene catalogues were highly associated with immune system, more specifically with cytokine signalling. Therefore, we selected annotated genes: *IL10*, *LIF*, *FOS*, *JUNB*, *IL6*, and *SOCS3* from common pathway "*Interleukin-4 and Interleukin-13 signaling*" (R-HAS-6785807) as prioritized gene candidates for expression levels quantification by qPCR. Interaction network of selected gene candidates is presented in Figure 1.

Gene expression validation. No significant correlation was detected between expression levels of selected genes and studied groups. Results of statistical analysis are presented in Table 3. However, trend of lower *LIF* expression levels ($p=0.083$) is possible to observe in adenomyosis cases *vs.* controls.

Literature screening, bioinformatics analysis and standardization of nomenclature across studies provided new gene candidates with a potential association in endometrial receptivity in adenomyosis. Despite validation experiment did not provide significant differentially expressed prioritized genes in adenomyosis or endometriosis groups when compared with controls, trend of lower (Figure 2) *LIF* mRNA expression levels ($p = 0.083$) was possible to observe in adenomyosis vs. controls. This gene was shown to be dysregulated and associated with interfered endometrial receptivity in adenomyosis cases by others (16). Moreover, *LIF* was enriched in our study also in other pathways, including "Interleukin-10 signalling", "IL-6-type cytokine receptor ligand interactions", "Interleukin-6-family signalling", "Cytokine signalling in immune system" and "Signalling by interleukins". From our validation experiment we conclude that "Interleukin-4 and Interleukin-13 signalling" may not be altered in adenomyosis or endometriosis. However, our sample number size was small. In the future, our hypotheses will be verified in larger number of samples. Additionally, genome-wide profiling may provide better insight of potential dysregulated mechanism in eutopic endometrium during WOI in women with adenomyosis.

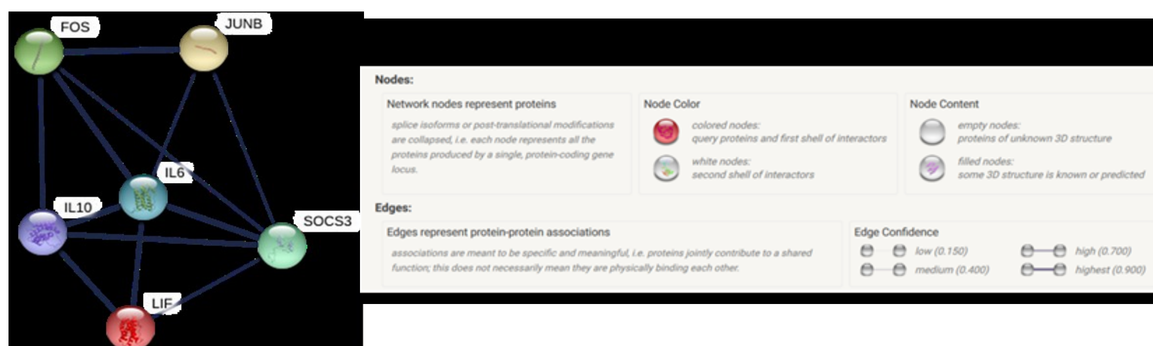

Figure 1: Interaction network of proteins whose genes were selected for expression analysis.

Table 1: List of genes, associated with adenomyosis and endometriosis retrieved through literature extraction. Gene symbols were edited according to the HGNC nomenclature.

ADENOMYOSIS	ENDOMETRIOSIS
MIR21, KLF12, NR4A1, IL10, HOXA10, LIFR, LIF, ITGB3, SPP1, TMPRSS11B, CHD5, SST, SPC25, ERCC6L, AKR1B10, CDKN3, ATP1A2, MB, KCNA4, MMP20, FNDC1, TUBAL3, SPINK2, COL11A1, ATP12A, CLDN4, C15orf62, SCGB2A2, TCN1, GPR78, CACNA1E, CYP3A7.	*PLEK, HOXA9, LAMA3, BST2, DLG5, SLA, COL12A1, PER1, SLC44A2, C1QTNF6, CDK5R1, EDNRB, S100A8, LTB4R2, PTAFR, GZMA, SAP30L, ALPI, TRIM15, SCGB2A2, TRPM6 , CRISP3, POMZP3, DEPP1, POMZP3, SEMA3C, GUCY1B2, ITGAL, DYNLL1, GNAZ, DBP, SOS1, MAP3K8, SLC6A6, BUB1B, SLC22A1, CCNK, EZR, ARF1, APOE, WT1, ATP7B, SLC1A1, SLC1A4, PTEN, SLC5A2, SLC6A7, ABCG1, INSRR, PTPN11, PPP3R1, EFNA4, SLC1A1, CYP3A5, EIF1, PAX8, ABCC3, ADGRF1, AIMP1, VEGFA, SLC15A4, GUCY1B1, YBX1, YBX1P2, MET, DST, RNF150, PCSK5, PRRC2C, SON, NFAT5, LUZP1, LONRF2, THRAP3, MUC7, AFF4, RIF1, MAP4, PLEKHA2, LPP, FOS, FOSB, EGR1, AOC1, CTSW, CCL3, CCL3L1, CCL3L3, CCN1, ACKR1, C1QA, CST7, ZFP36, JUNB, CDA, RSRP1, SMG1, NEAT1, TRPM6, MMP26, KRIT1, PCYOX1, ENPP3, CRISP3, DDX17, AMY1A, AMY2A, AMY2B, DNAJC3, CWH43, CASP5, CELF1, ABCB11, VDAC1P1, ZIC2, CA1, PRIM2, IFNA21, TGFB3, SCG2, VHL, S100A3, RAB9BP1, TAF6L, SERPINB8, RIN1, MAPK8, LCK, SHB, NCR1, CCL8, MALL, FOSB, IL6, ATF3, EGR3, HPCAL4, RGS1, CXCL2, FOS, NR4A3, NR4A1, KRT5, CCN1, EGR1, SOCS3, EGR2, CRABP1, FMN2, GALP, HACD1, CA12, SP3P, PITX1, AGT, CCBE1, KCNK2, LRRD1, DDIT4L, KRTAP19-2, MIR196A1, MIR196A2, MIR3196, MIR135A1, MIR5585, MIR194-2, MIR1915, MIR138-1, MIR138-2, MIR921, MIR4425, MIR365B, MIR542, MIR4251, MIR26B, MIR219B, MIR4252, MIR4723, MIR548AA2, MIR548AP, MIR548T, MIR505, MIR4254, MIR3686, MIR22, MIR339, MIR374B, RBBP4, PDHB, RNH1, VIM, TUBA1C, PRDX6, MYL12A, VIM, EIF4A1, EIF4A2, ANXA5, KRT18, HSP90B1, YWHAE, ANXA2, ACO2, GSN, IMMT, CORO1B, CCT8.*

Table 2: Top 10 pathways determined by Reactome using gene catalogue of adenomyosis and endometriosis. Genes from "Interleukin-4 and Interleukin-13 signalling" pathway (marked with dark grey) were selected for validation.

	Pathway name	Entities pValue	Entities FDR	Submitted entities found
Adenomyosis	RUNX1 regulates transcription of genes involved in interleukin signaling	2.9E-04	0.107	LIFR
	RUNX3 Regulates Immune Response and Cell Migration	7.7E-04	0.125	SPP1
	Degradation of the extracellular matrix	1.2E-03	0.125	COL11A1; SPP1; MMP20
	MECP2 regulates transcription of neuronal ligands	1.4E-03	0.125	SST
	Ion transport by P-type ATPases	2.7E-03	0.196	ATP1A2; ATP12A
	IL-6-type cytokine receptor ligand interactions	4.9E-03	0.296	LIF; LIFR
	Interleukin-4 and Interleukin-13 signaling	8.9E-03	0.317	IL10; LIF
	Interleukin-10 signaling	1.0E-02	0.317	IL10; LIF
	Separation of Sister Chromatids	1.1E-02	0.317	ERCC6L; TUBAL3; SPC25
Endometriosis	Interleukin-10 signaling	2.3E-04	0.284	IL6; CCL3L1; CCL3L3; PTAFR; CCL3; CXCL2
	Neutrophil degranulation	1.0E-03	0.476	CDA; AOC1; GSN; ANXA2; SLC44A2; CRISP3; PTAFR; ITGAL; DYNLL1; CXCL2; PRDX6; DNAJC3; BST2; CCT8; KCNK2; SLC15A4; S100A8
	Digestion of dietary carbohydrate	1.1E-03	0.476	AMY2A; AMY2B; AMY1A
	Interleukin-4 and Interleukin-13 signaling	3.6E-03	0.856	SOCS3; IL6; VIM; FOS; JUNB; VEGFA; HSP90B1
	Digestion	4.0E-03	0.856	AMY2A; AMY2B; AMY1A; ALPI
	Post-translational protein phosphorylation	4.6E-03	0.856	DNAJC3; IL6; APOE; CCN1; SCG2; HSP90B1
	Proton/oligopeptide cotransporters	8.2E-03	0.856	SLC15A4
	Type I hemidesmosome assembly	8.8E-03	0.856	DST; LAMA3; KRT5
	Digestion and absorption	1.2E-02	0.856	AMY2A; AMY2B; AMY1A; ALPI
	Reversible hydration of carbon dioxide	2.7E-02	0.856	CA12; CA1

Table 3: Statistical analysis of qPCR data between studied groups.

Compared study groups	Statistical test	*IL6* mRNA fold change	*LIF* mRNA fold change	*IL10* mRNA fold change	*SOCS3* mRNA fold change	*JUNB* mRNA fold change	*FOS* mRNA fold change
Endometriosis *vs.* controls	Mann-Whitney U	14.000	14.000	12.000	13.000	14.000	12.000
	Wilcoxon W	24.000	24.000	22.000	23.000	24.000	22.000
	Z	-0.340	-0.340	-0.679	-0.510	-0.340	-0.679
	Asymp. Sig. (2-tailed)	0.734	0.734	0.497	0.610	0.734	0.497
Adenomyosis *vs.* controls	Mann-Whitney U	26.000	13.000	14.000	14.000	25.000	28.000
	Wilcoxon W	54.000	41.000	42.000	42.000	53.000	64.000
	Z	-0.231	-1.736	-1.620	-1.620	-0.347	0.000
	Asymp. Sig. (2-tailed)	0.817	0.083	0.105	0.105	0.728	1.000
Endometriosis *vs.* adenomyosis	Mann-Whitney U	14.000	9.000	8.000	10,000	14.000	11.500
	Wilcoxon W	42.000	37.000	36.000	38,000	42.000	21.500
	Z	0.000	-0.945	-1.134	-0.756	0.000	-0.474
	Asymp. Sig. (2-tailed)	1.000	0.345	0.257	0.450	1.000	0.636

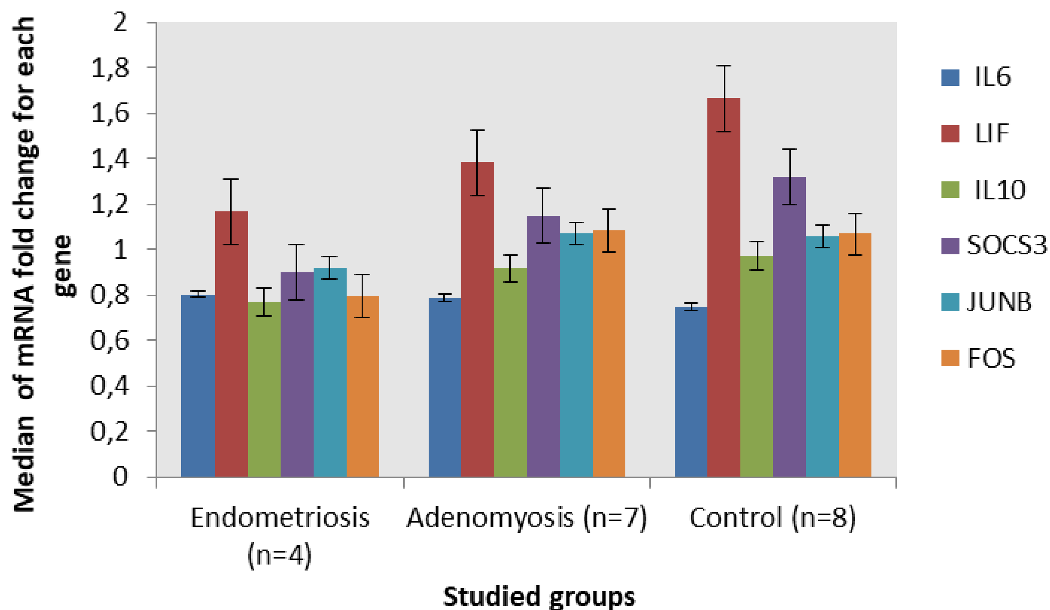

Figure 2: LI6, LIF, IL10, SOCS3, JUNB and FOS mRNA expression levels in studied groups

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P6

SEQUENCE ANALYSIS OF *IRF6* GENE IN FAMILIES WITH OROFACIAL CLEFTING

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POVZETEK

Orofacialne shize (OFC) so najpogostejša oblika prirojenih nepravilnosti obraza in ustne votline. Večina shiz je nesindromskih (izolirane oblike), pojavljajo pa se tudi v sklopu različnih sindromov (npr. Van der Woude sindrom). Nesindromske OFC nastanejo zaradi interakcije med več genetskimi in okoljskimi dejavniki. V nekaterih študijah so dokazali povezavo med OFC in mutacijami v genu *IRF6*. Raziskavo smo izvedli z namenom, da bi preverili mutacijski profil vseh eksonov (in njihovih mej z introni) v genu *IRF6* pri slovenskih bolnikih s potrjeno diagnozo OFC in pozitivno družinsko anamnezo te malformacije. V študijo (odobreno s strani Komisije za medicinsko etiko: 57/02/13) smo vključili štiriindvajset bolnikov z OFC ter njihove sorodnike. V DNA vzorcih bolnikov z OFC in njihovih sorodnikov smo s pomočjo sekvenciranja po Sangerju identificirali šest mutacij, in sicer eno mutacijo spojitvenega mesta (rs7552506), dve intronski (rs2235375, rs2235373), eno nesmiselno (CM022409; Arg412X) in dve sinonimni mutaciji (rs2013162, rs763754100). Posledice odkritih mutacij smo analizirali *in silico* z uporabo različnih bioinformatičnih orodij. Ugotovili smo, da se genotip v primeru večine polimorfizmov izraža fenotipsko variabilno. Izjema je nesmiselna mutacija Arg412X (značilna za Van der Woudeov sindrom), ki izkazuje monogenski avtosomno dominantni vzorec dedovanja. Pomembno odkritje je tudi sinonimna mutacija rs763754100, ki je v naši kohorti bolnikov veliko pogostejša kot v populaciji zdravih posameznikov iz baze 1000 Genomes. Ostale štiri odkrite mutacije najverjetneje sodelujejo pri poligenskem dedovanju OFC. Med polimorfizmi rs2235375, rs7552506 in rs2013162 smo ugotovili tudi indikacije za vezavno neravnovesje.

Ključne besede: orofacialne shize, *IRF6*, polimorfizmi, študija družin

ABSTRACT

Orofacial clefts (OFC) are the most common form of congenital anomalies of the face and oral cavity. Most clefts are non-syndromic (isolated forms), but they also occur as part of various syndromes (e.g. Van der Woude syndrome). Non-syndromic OFC are caused by the interaction between several genetic and environmental factors. Some studies have demonstrated a link between OFC and mutations in the *IRF6* gene. This study was performed in order to examine the mutation profile of all exons (and their intron boundaries) of the *IRF6* gene in Slovenian patients with a confirmed diagnosis of OFC and the positive family anamnesis of it. The study (approved by National Medical Ethics Committee: 57/02/13) included twenty-four OFC patients and their relatives. Using Sanger sequencing, six mutations were identified in the DNA samples of OFC patients and their relatives, namely one splice site mutation (rs7552506), two intronic (rs2235375, rs2235373), one

nonsense (CM022409; Arg412X) and two synonymous mutations (rs2013162, rs763754100). The consequences of the detected mutations were predicted *in silico* using various bioinformatic tools. The genotype was found to be phenotypically variable in the case of most polymorphisms. An exception is the mutation Arg412X (characteristic of Van der Woude syndrome), which has monogenic autosomal dominant inheritance. Another significant finding is the synonymous mutation rs763754100, which is much more common in our patient cohort than in the global population of healthy individuals from the 1000 Genomes database. The other four detected mutations most likely participate in the polygenic inheritance of OFC. We also found evidence for linkage disequilibrium between polymorphisms rs2235375, rs7552506 and rs2013162.

Key words: orofacial clefts, *IRF6*, polymorphisms, family study

POSTERS – BIOTECHNOLOGY

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P7

PREPARATION OF gRNA CONSTRUCTS FOR MULTIPLEX CRISPR-Cas9

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POVZETEK

Sistem CRISPR-Cas9 se že nekaj let uspešno uporablja za urejanje genoma številnih organizmov, od bakterij, kvasovk in rastlin, do živali in ljudi. Endonukleazo Cas9 do tarčnega mesta na genomu vodi kratko zaporedje RNA, imenovano vodeča RNA (gRNA). Prepoznavanje tarčnega mesta na genomu poteka preko parjenja baz med gRNA, vezano na Cas9, in specifičnim odsekom na genomski DNA. Uvajanje več sprememb v genom je zaenkrat še relativno dolgotrajen proces, pri katerem vsaka posamezna mutacija zahteva svoj cikel transformacije in selekcije. Zato smo, z enostavno metodo uporabe restrikcije in ligacije, pripravili konstrukte z do 6 različnimi gRNA v plazmidu. Zaporedja gRNA, ki smo jih izbrali, omogočajo ciljanje genov, ki sodelujejo pri sintezi aminokislin v kvasovki *Saccharomyces cerevisiae* in posledično omogočajo uvajanje ali izničevanje avksotrofij, ki so pomembno pomagalo pri uporabi kvasovke kot genetskega modelnega organizma. Razvita metoda se potencialno lahko uporabi za nadgradnjo že obstoječega sistema CRISPR-Cas9 za hkratno uvajanje več genomskih modifikacij na poljubnih lokusih.

Ključne besede: editiranje genoma, večkratni CRISPR-Cas9, gRNA*, Saccharomyces cerevisiae*

ABSTRACT

In recent years CRISPR-Cas9 system has been successfully applied for genome editing in a variety of species, from bacteria, yeast, plants, to animals and humans. In the nucleus, the Cas9 endonuclease is guided to the target site by a short RNA molecule, called guide RNA (gRNA). Target site recognition depends on specific base pairing between gRNA and the specific genome sequence. However, using the existing methods for gene editing, introducing multiple genetic modifications is still a relatively time-consuming process. As the majority of currently available CRISPR-Cas9 based methods can modify only one target site at a time. We therefore prepared plasmids with up to 6 different gRNA constructs, using standard restriction and ligation methods. The selected gRNA sequences were designed to target genes involved in amino acid biosynthesis in yeast *Saccharomyces cerevisiae,* enabling introduction of auxotrophies, which are an important feature of yeast as a genomic model organism.. Our method can potentially improve the existing CRISPR-Cas9 system so that it can be used for simultaneus introduction of multiple genetic modifications into any loci of the genome.

Key words: genome editing, multiplex CRISPR-Cas9, gRNA, *Saccharomyces cerevisiae*

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P8

OPTIMIZATION OF CTLA4 BINDING IgG1 ANTIBODY USING MOLECULAR MODELING AND DYNAMICS SIMULATIONS

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POVZETEK

Melanom je zaradi imunogene narave pogosto obdan z regulatornimi T celicami (Treg), ki na svoji površini izražajo s citotoksičnimi limfoctiti T povezani protein 4 (CTLA4). S tem mehanizmom melanom utiša imunski odziv v svojem mikrookolju. Novejše raziskave, ki obravnavajo mehanizem delovanja IgG1 protiteles z vezavo na CTLA4, izpostavljajo Treg celice in njihovo celično smrt kot glavni mehanizem protirakavega delovanja. Vlogo pri tem ima domena Fc IgG1 protiteles, ki posreduje citotoksični učinek na celice Treg prek vezave na FcγR. Slednji se nahajajo na površini efektorskih celic imunskega sistema. Za doseganje terapevtske učinkovitosti je ključna vezava določenih podtipov FcγR na regijo Fc IgG1 protiteles. Z uporabo homolognega modeliranja smo skonstruirali IgG1 protitelo. Iskanje po literaturi je ponudilo idejo za možno mutacijo, ki bi povzročila višjo vezavo želenih FcγRIIa in FcγRIIIa. Afiniteta vezave izbranih FcγR je bila ocenjena po izvedbi molekularne dinamike s programom CHARMM z analizo povprečnega števila vodikovih vezi po 16 ns s programom Visual Molecular Dynamics (VMD). Mutacija IgG1 protitelesa G237A je povzročila večje povprečno število vodikovih vezi med Fc regijo in vsemi tremi preučenimi FcγR, kar ni zaželeno, saj smo želeli višje število le za aktivacijska FcγR IIa in IIIa in ne tudi za inhibitorni FcγR IIb. V prihodnosti želimo omenjene interakcije analizirati tudi z bolj striktnimi metodami za izračun protein-ligand vezavne energije.

Ključne besede: Fcγ receptor, molekularno modeliranje, molekularna dinamika, IgG1 protitelo

ABSTRACT

Melanoma is a very immunogenic cancer that often surrounds itself with regulatory T cells (Treg) that express on their surfaces the cytotoxic T-lymphocyte-associated protein 4 (CTLA4). This allows melanoma to dampen the immune response in its microenviroment. Recent studies of IgG1 antibodies that bind to CTLA4 suggest a novel therapeutic strategy where the key to efficient therapy is depleting the Treg cells. The Fc region of the IgG1 antibody plays a role in this strategy as it mediates the cytotoxic effect on Treg cells via binding to the FcγR found on these immune effector cells. To achieve the best therapeutic efficacy, subtypes of FcγRIIa and FcγRIIIa need to bind to the Fc region. Using in silico homology modeling we modeled a complete IgG1 antibody. Insight into a possible mutation that would increase binding of the correct FcγR subtypes was found in literature. The binding affinity was assessed after molecular dynamics using CHARMM by calculating the average number of hydrogen bonds during a 16ns period with the VMD program. Mutation G237A of the IgG1 antibody shows a higher average hydrogen bond number across all FcγR, which is not desired as we expected only the increase of the activatory FcγR IIa and IIIa and not the inhibitory

FcγRIIb. In the future we wish to analyze these interactions using more stringent methods for calculating protein-ligand binding energy.

Key words: Fcγ receptor, molecular modeling, molecular dynamics, IgG1 antibody

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P9

INFECTION OF HOP PLANTS (*Humulus lupulus*) WITH *Verticillium nonalfalfae* INDUCES CHANGES IN THE CONTENT OF PHENOLIC COMPOUNDS AND miRNA EXPRESSION

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POVZETEK

Do danes je malo znanega o obrambnih mehanizmih hmelja pred glivnim patogenom *Verticillium nonalfalfae*, ki je glavni povzročitelj hmeljeve uvelosti. Cilj naše študije je proučevanje odziva hmelja na ravni mikro RNA (miRNA) in fenolnih spojin, saj pretekle študije kažejo, da so le-te vpete v odziv rastlin na številne biotske in abiotske dejavnike stresa. Patosistem hmelja in *V. nonalfalfae* predstavlja odličen model za preučevanje obrambnih odzivov s fenoli in miRNA med patogenezo verticilijske uvelosti. V okviru študije smo 1) spremljali kolonizacijo glive, 2) določili vsebnost fenolnih spojin in 3) identificirali diferenčno izražene miRNA pri občutljivem (Celeia - CE) in odpornem (Wye Target - WT) kultivarju hmelja po okužbi z *V. nonalfalfae*. Profil kolonizacije nakazuje na hitro kolonizacijo korenin pri obeh sortah, ter kontinuirano zmanjševanje relativne količine glive tekom eksperimenta v odpornem kultivarju, medtem ko se pri občutljivem kultivarju pojavi re-kolonizacija korenin in razrast glive v stebelno tkivo 12. dan po okužbi (DPI). Prav tako profil fenolnih spojin nakazuje na povečanje skupnih analiziranih fenolov v zgodnji fazi okužbe pri obeh kultivarjih. Z analizo miRNA-Seq podatkov koreninskih vzorcev iz 1.DPI smo identificirali 182 kandidatnih miRNA, od teh je 128 že znanih in pripadajo 30 različnim miRNA družinam. V CE smo identificirali 14 (9 znanih in 5 novih), v WT pa 12 (11 znanih in 1 nova) diferenčno izraženih miRNA. Na okužbo odzivne miRNA smo validirali z uporabo RT-qPCR metode ter opravili identifikacijo in računalniško funkcijsko analizo tarčnih miRNA transkriptov hmelja.

Ključne besede: hmelj, verticilijska uvelost, fenolne snovi, mikro RNA

ABSTRACT

To date, little is known about the defense mechanisms of hops (*Humulus lupulus*) against the fungal pathogen *Verticillium nonalfalfae*, a major causative agent of Verticillium wilt. Previous studies suggest the role of miRNAs and phenolic compounds in plant responses to biotic stress, therefore a unique pathosystem of hops and *V. nonalfalfae* represents a great model to study such defense responses during the pathogenesis of Verticillium wilt. The aim of our study was to 1) monitor *V. nonalfalfae* colonization, 2) determine phenolic content, and 3) identify differentially expressed miRNAs as a response to *V. nonalfalfae* infection in susceptible Celeia (CE) and resistant Wye Target (WT) hop cultivars.Following rapid colonization of roots in both cultivars, a fungal relative amount continuously declines in the resistant cultivar throughout the experiment, whereas in the susceptible cultivar, a re-colonization of roots and spread of the fungus into the stems occur at 12thday post-inoculation (DPI). Additionally, phenolic compounds profile shows an increase in total analyzed phenols in early stage of infection in both cultivars. In a miRNA-Seq analysis of WT and CE root samples from 1 DPI, we identified 182 miRNA candidates, of those,128 miRNA candidates mapped to 30 known miRNA gene families. We identified 14 (9 known and 5 novel) and 12 (11 known and 1 novel) differentially expressed miRNAs in CE and WT cultivar, respectively. The identified responsive hop miRNAs were validated using RT-qPCR and identification and computational functional analysis of miRNA target transcripts was performed.

Key words: hop, Verticillium wilt, phenolic compounds, micro RNA

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P10

MATURATION AND CHROMOSOME DOUBLING OF *Brassica napus* HAPLOID EMBRYOS

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IZVLEČEK

Oljna ogrščica (*Brassica napus* L.) je svetovno pomembna oljnica, ki je zaradi svoje kemične sestave izjemno pomembna v industriji in v prehrani. S tehniko izolacije mikrospor lahko regeneriramo haploidne in podvojene haploidne rastline, katerih cilj je ustvariti homozigotne genotipe oljne ogrščice (ali drugih rastlinskih vrst) iz heterozigotnih starševskih linij v samo eni generaciji. Ker je spontano podvojevanje haploidnih embrijev oljne ogrščice v kulturi redko, se za podvojevanje kromosomov najpogosteje uporablja sredstvo kolhicin, ki moti mitozo z zaviranjem tvorbe niti delitvenega vretena. To prepreči normalno razdvajanje kromosomov na celične pole, zaradi česar se število kromosomov v celici podvoji. V raziskavi smo preizkusili različne metode za uspešno regeneracijo embrijev in podvojitev njihovih kromosomov. Ugotovili smo, da metoda izsuševanja ni primerna za regeneracijo rastlin oljne ogrščice, za primerno sredstvo pa se je izkazalo tretiranje z buthionine sulfoximine (BSO). Do podvojitve kromosomov je prišlo pri rastlinah tretiranih z abscizinsko kislino in pri rastlinah tretiranih s kolhicinom.

Ključne besede: *Brassica napus* L., mikrospore, embriogeneza, podvojevanje kromosomov

ABSTRACT

Oilseed rape (*Brassica napus* L.) is a worldwide important oilseed plant and because of its chemical composition, it is important in industry and food production. Isolated microspore culture technique is used to generate haploid and doubled haploid plants, which aim to create homozygous *Brassica napus* L. genotypes from heterozygous parental lines in just one generation. Since spontaneous chromosome doubling of haploid embryos is rare in *Brassica napus* microspore culture, colchicine is the most commonly used compound for chromosome doubling. It disrupts mitosis by inhibiting the formation of spindle fibers and therefore disturbing normal polar chromosomal migration resulting in chromosome doubling. In the study, we tested different methods for successful regeneration of embryos and doubling of chromosomes. We discovered that the desiccation was a not suitable method for the regeneration of oilseed rape plants, however treatment with buthionine sulfoximine (BSO) proved to be an appropriate agent. Chromosome doubling was observed in plants treated with abscisic acid and in plants treated with colchicine.

Key words: *Brassica napus* L., microspores, embryogenesis, chromosome doubling

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P11

SPEED BREEDING OF OILSEED RAPE (*Brassica napus* L.) FOR MICROSPORE EMBRYOGENESIS

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POVZETEK

Genetske raziskave ter pridobivanje novih sort je lahko dolgotrajen proces, zato raziskovalci iščejo inovativne načine hitrejšega razvoja rastlin. 'Speed breeding' je eden izmed teh načinov. Ta s pomočjo različnih stresnih dejavnikov, kot so osvetlitev, gostota setve in temperatura pospeši fiziološke faze raziskovanih rastlin. S pomočjo te tehnike smo hoteli v čim krajšem času pridobiti cvetne brste oljne ogrščice ter s tem pospešiti postopek pridobivanja mikrospor, iz katerih kasneje dobimo haploide in podvojene haploide. Tehnike izolacije mikrospor so pri križnicah standardizirane. Namen poskusa je bil ugotoviti kako vplivata gostota sajenja in perioda osvetlitve na hitrost pojavljanja brstov ter kakšen vpliv ima to na razvoj embrijev. Rastline so bile osvetljene z lučmi LED 22 ali 16 ur ter sajene v lonce (premer 12,5 cm) z 1 ali 3 rastlinami. Ob pojavu prvih dveh cvetov so se izolirale mikrospore. Izolacija in gojenje mikrospor je potekala po postopku opisanem v Custers (2003). Po izolaciji smo izmerili koncentracijo in preverili živost izoliranih mikrospor s pomočjo metode FDA. Ko so se razvili embriji, so bili prešteti. Po štetju se je v gojišče dodala ABA (3mg/L), nato so bili po enem dnevu embriji prestavljeni na trdo B5 gojišče, kasneje so se rastline aklimatizirale. Podatki nakazujejo, da hitreje zacvetijo rastline pod 22 urno osvetlitvijo. Povprečen čas od setve do pojava prvih 2 cvetov oziroma izolacije je bil 77dni. Največ mikrospor, posledično tudi embrijev, se je razvilo 50-60 dni po vzorčenju.

Ključne besede: *Brassica napus* L., mikrospore, mikrosporna embriogeneza

ABSTRACT

Genetic research and the acquisition of new varieties can be a long process, so researchers are looking for innovative ways to grow plants faster. Speed breeding is one of these ways. This method, through various stress factors such as lighting, sowing density and temperature, accelerates the physiological stages of the studied plants. With the help of this technique, we wanted to obtain flower buds of oilseed rape as soon as possible, thus speeding up the process of obtaining microspores from which haploids and doubled haploids were later obtained. Microspore isolation techniques are standardized for Brassica species. The purpose of the experiment was to determine how the density of planting and the period of illumination affect the speed of bud emergence and what impact it has on embryo development. Plants were illuminated with LED lights for 22 or 16 hours and planted in pots (diameter 12,5 cm) with 1 or 3 plants. When the first two flowers appeared, the microspores were isolated. The isolation and cultivation of the microspores were carried out according to the protocol published by Custers (2003). After isolation, the percentage of live microspores was checked. When embryos developed, they were counted. After counting, ABA (3 mg / L) was added to the culture medium, then after one day the embryos were transferred to solid B5 medium, developed plants were acclimatized. The data suggest that plants bloom faster under 22 hours of exposure to light. The average time from sowing to the appearance of the first 2 flowers or

isolation was 77 days. Most microspores, and consequently embryos, developed 50-60 days after sampling.

Key words: *Brassica napus* L., microspore, microspore embryogenesis, speed breeding

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P12

STUDYING POLARITY OF DIFFERENT VIROIDS AND THEIR COMBINATIONS IN INFECTED HOP PLANTS

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POVZETEK

Viroidi so majhne, visoko infektivne, enoverižne, krožne molekule RNA. Preučevanje okuženih rastlin z viroidi se v glavnem osredotoča na okužbe z eno vrsto viroida. Hmelj (*Humulus lupulus* L.) je gostitelj štirim viroidom, v našem delu pa smo raziskovali polarnost in nivo akumulacije viroidov HLVd, CBCVd in HSVd pri hkratnih okužbah hmelja. Rezultati RT-qPCR in podatki RNA-seq so pokazali, da se viroidi v rastlini razmnožijo do nivoja zasičenja, ki je verjetno pogojen z omejenimi viri energije gostitelja. Pri hkratnih okužbah smo pokazali tudi na antagonizem med nekaterimi patogeni, zlasti med viroidoma CBCVd in HSVd. Izkaže se, da je viroid HSVd najbolj, viroid CBCVd pa najmanj občutljiv na prisotnost drugega viroida. Podatki RNA-seq kažejo tudi na to, da pri viroidih HLVd in CBCVd prevladuje (−) oblika, kar je zanimivo, kajti nekateri podatki iz literature pravijo ravno nasprotno. Glede polarnosti pa rezultati RT-qPCR niso konsistentni, sploh pa so manj zanesljivi, zaradi določene stopnje biološke variabilnosti, kar kaže na to, da je v podobnih eksperimentih potrebno analizirati večje število rastlin. Menimo, da bi morali za študij polarnosti, nivojev okužb in interakcij viroid-rastlina uporabiti alternativne metode, ki temeljijo na neposrednem štetju molekul viroidnih RNA.

Ključne besede: hmelj, viroidi, polarnost, interakcije viroidi-rastlina, bioinformatska analiza, RT-qPCR

ABSTRACT

Viroids are small, highly infectious, single-stranded, circular RNA molecules. The study of viroid infected plants mainly focuses on infections with one type of viroid. Hop plants (*Humulus lupulus* L.) host four viroids however, in our work we investigated the polarity and accumulation of HLVd, CBCVd, and HSVd in simultaneous hop infections. RT-qPCR results and RNA-seq data showed that the viroids amplify in hop to the level of saturation, which is probably limited by host resources. Antagonism between some pathogens seems to be present as well, especially between CBCVd and HSVd viroids. It turns out that HSVd is the most and CBCVd is the least sensitive to the presence of another viroid. RNA-seq data also indicate that HLVd and CBCVd (−) strand accumulation exceeds (+) strand, which contradicts to some literature data. In terms of polarity, however, the RT-qPCR results are less consistent, due to biological variability, suggesting that larger numbers of plants need to be analysed in similar experiments. We believe that alternative methods based on the direct counting of viroid RNA molecules should be used to study the polarity, infection levels, and viroid-plant interactions.

Key words: hop, viroids, polarity, viroids-plant interactions, bioinformatic analysis, RT-qPCR

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CHARACTERIZATION OF SEVEN BREEDING LINES OF MEDICAL CANNABIS (*Cannabis sativa* L.)

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POVZETEK

Cannabis sativa L. je dvodomna enoletna cvetoča rastlina, ki spada v družino *Cannabiaceace*. Ima zelo široko uporabno vrednost, saj se uporablja za pridobivanje vlaken, semen, olja iz semen, kanabinoidov, za pridobivanje biomase in drugo. Prav tako ima potencialno uporabno vrednost v farmacevtske namene. Glavne aktivne kemične spojine so iz skupine kanabinoidov, za ketere so dokazali, da imajo terapevtski učinek. Med temi so najpomembnejši THCA (delta-9 tetrahidrokanabinolna kislina), CBDA (kanabidiolska kislina) in CBNA (kanabinolna kislina). Da bi lahko konopljo uporabljali v terapevtske namene, je potrebno požlahtniti sorte z določeno koncentracijo kanabinoidov.

V raziskavi smo kvantificirali kanabinoide iz sedmih žlahtniteljskih linijah medicinske konoplje *Cannabis sativa* L. s HPLC metodo (ang. high-pressure liquid chromatography). V analizo smo vključili 46 rastlin žlahtniteljske linije MX-CBD-11, 49 rastlin žlahtniteljske linije MX-CBD-707, 5 rastlin žlahtniteljske linije CBD-MX-81, 5 rastlin žlahtniteljske linije CBD-MX-91, 9 rastlin žlahtniteljske linije MIX-MX-40, 5 rastlin žlahtniteljske linije THC-MX-10 in 3 rastline žlahtniteljske linije THC-MX-15. Vseh 122 rastlin je bilo analizirano tudi z označevlci 16 SSR, ki so jih razvili Geo in sod. (2014). Vse žlahtniteljske linije, ki so bile vključene v raziskavo, so last MGC Pharma (UK) Ltd.

Ključne besede: *Cannabis sativa* L, kanabinoidi, označevalec SSR, žlahtnjenje

ABSTRACT

Cannabis sativa L. is a dioecious, annual flowering species belonging to the family *Cannabiaceace*. It is multi-use crop, able to provide fiber, seeds and seed oil, cannabinoids, biomass. It is alsouseful for pharmaceutical applications. The main chemically active compounds found in cannabis are cannabinoids, which have a proved therapeutic potential. The most abundant cannabinoids are THCA (Tetrahydrocannabinolic acid), CBDA (cannabidiolic acid) and CBNA (cannabinolic acid). To benefit the therapeutic properties of cannabis, breeding new varieties with tailored cannabinoid content is needed.

In our study, we quantified cannabinoids in seven breeding lines of medical *Cannabis sativa* L. with HPLC (high-pressure liquid chromatography). The analyses were done on 46 plants of breeding line MX-CBD-11, 49 plants of breeding line MX-CBD-707, 5 plants of breeding line CBD-MX-81, 5 plants of breeding line CBD-MX-91, 9 plants of breeding line MIX-MX-40, 5 plants of breeding line THC-MX-10 and 3 plants of breeding line THC-MX-15. All these 122 plants were also analyzed with 16 SSR markers developed by Gao et al. (2014). All breeding lines included in the study are owned by MGC Pharma (UK) Ltd.

Key words: *Cannabis sativa* L., cannabinoids, SSR markers, breeding

POSTERS – POPULATION GENETICS

Katja Molan

P14: GUT MICROBIOTA OF WOMEN WITH GESTATIONAL DIABETES AND FOETAL MACROSOMIA

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GUT MICROBIOTA OF WOMEN WITH GESTATIONAL DIABETES AND FOETAL MACROSOMIA

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POVZETEK

Črevesna mikrobiota je pomemben dejavnik človeškega zdravja. Disbioze so povezali z različnimi boleznimi, tudi z gestacijskim diabetesom (GDM). GDM predstavlja dejavnik tveganja za makrosomijo in za razvoj debelosti pri otroku ter razvoj diabetesa tako pri otroku kot pri materi. Predpostavili smo, da obstajajo razlike v mikrobioti nosečnic z GDM, ki so rodile makrosomne otroke, v primerjavi z nosečnicami, ki so rodile otroka z normalno porodno težo. Izmed47 vključenih nosečnic smo jih izbrali 20 in izvedli NGS 16S metagenomsko analizo DNA, izolirane iz fecesa v 2. in 3. trimesečju nosečnosti. Osem nosečnic je rodilo makrosomnega otroka. Pri skupini nosečnic z GDM in makrosomnim otrokom je analiza pokazala nižji odstotek *Firmicutes* (p=0,028) v 2. trimesečju nosečnosti kot pri skupini nosečnic, ki so rodile otroka z normalno težo, in sicer na račun Clostridia (p=0,028). Na nivoju družine bakterij smo opazili več statistično značilnih razlik v obeh trimesečjih (npr. nižji odstotek *Alcaligenaceae*, *Moraxellaceae*, *Xanthomonadaceae* pri nosečnicah z makrosomnim otrokom), kar nakazuje na persistentne spremembe povezane z makrosomijo. Nosečnice z makrosomnim otrokom so bile starejše (37 ± 3 v primerjavi z 31 ± 4 leta, p = 0,008), z višjimi vrednostmi serumskih trigliceridov (2,6±0,2 v primerjavi z 1,9±0,5 mmol/l, p=0,002), medtem ko sta bili glikemija in pridobljena telesna teža med nosečnostjo primerljivi v obeh skupinah.

Ključne besede: črevesna mikrobiota, gestacijski diabetes (GDM), makrosomija, 16S NGS

ABSTRACT

Gut microbiota is crucial for human health. Dysbiosis has been associated with various diseases, among them also gestational diabetes (GDM). GDM is a risk factor for foetal macrosomia, child's obesity and diabetes mellitus of the mother and the child later in life. We hypothesized that differences in the gut microbiota exist between the GDM-women who delivered macrosomic babies and the GDM-women who delivered normal weight babies. Among 47 recruited GDM-women, 20 were selected for 16S metagenomic DNA analysis of faecal gut microbiota by NGS performed in the second and third trimester. Eight of GDM-women delivered a macrosomic baby. The 16S sequencing analysis revealed that GDM-women with macrosomic babies had a lower abundance of *Firmicutes* (p=0.028) driven by a lower abundance of Clostridia (p=0.028) in the second trimester. On the level of bacterial family, several significant differences were observed in both trimesters (e.g. lower *Alcaligenaceae*, *Moraxellaceae*, *Xanthomonadaceae* in GDM-women with macrosomic babies), indicating persistent changes associated with macrosomia. Regarding the phenotype, these women were older (37±3 vs. 31±4 years, p=0.008), had higher serum triglycerides (2.6±0.2 vs. 1.9±0.5 mmol/l, p=0.002), while glycaemic control and weight gain were comparable in both groups.

Key words: gut microbiota, gestational diabetes mellitus, macrosomia, 16S NGS

POSTERS – GENOMICS

Julia S. Gizatullina

P15: PREVALENCE OF ANTIBIOTIC RESISTANCE GENES AND CLASS I INTEGRONS AMONG AVIAN PATHOGENIC *Escherichia coli* STRAINS

Taja Jeseničnik

P16: NGS SEQUENCING AND BIOINFORMATIC ANALYSIS REVEALS MICRO RNAs IN PATHOGENIC PLANT FUNGI *Verticillium nonalfalfae*

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PREVALENCE OF ANTIBIOTIC RESISTANCE GENES AND CLASS I INTEGRONS AMONG AVIAN PATHOGENIC *Escherichia coli* STRAINS

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POVZETEK

Ptičja patogena bakterija *Escherichia coli* (APEC) je glavna povzročiteljica ptičje kolibaciloze v svetu in zaradi visoke stopnje obolevnosti in umrljivosti predstavlja velik gospodarski problem za perutninsko industrijo. Protimikrobna zdravila igrajo pomembno vlogo pri obvladovanju okužb z *E. coli*, vendar njihova nenehna uporaba vodi do pojava sevov odpornih proti tem zdravilom, vključno s sevi, ki so odporni proti več antibiotikom hkrati. Namen naše študije je bil ugotoviti razširjenost izbranih genov za odpornost proti antibiotikom med 28 sevi APEC, izoliranimi v obdobju 2016–2018 iz različnih organov piščancev s kolibacilozo. Preučevali smo gene, ki kodirajo β-laktamaze ($bla_{TEM\text{-},1}$, bla_{SHV}, $bla_{CTX\text{-}M}$, $bla_{OXA\text{-}1}$, $bla_{CMY\text{-}1}$), aminoglikozidno 2'-N-acetiltransferazo (*aacC2*) in regijo integronov razreda 1 (*intI*). S PCR smo lahko pomnožili gen $bla_{TEM\text{-}1}$ v 20 (71.4 %) sevih, drugi najpogostejšo najden je bil gen $bla_{CTX\text{-}M}$ (53.6 % sevov), medtem ko smo gen bla_{SHV} odkrili le v enem sevu. Genov $bla_{CMY\text{-}1}$ in $bla_{OXA\text{-}1}$ nismo našli med preučevanimi sevi. V 8 sevih (28.6 %) smo našli gen *aac2*. Kombinacijo $bla_{TEM\text{-}1}$ + $bla_{CTX\text{-}M}$ smo našli v 13 (46.4%) sevih in kombinacijo $bla_{TEM\text{-}1}$ + $bla_{CTX\text{-}M}$ + *aacC2* v 6 (21.4 %) sevih. V 8 (28.6 %) sevih smo odkrili fragmente integronov razreda 1 (velikost fragmentov je bila od 800 do 2000 bp) in dva od njih sta bila na konjugativnih plazmidih. Vsi pomnoženi fragmenti integronov razreda 1 so bili povezani s prisotnostjo genov za odpornost proti ampicilinu/cefalosporinu in v dveh primerih tudi z odpornostjo proti aminoglikozidom. Da povzamemo, naši podatki kažejo, da so sevi APEC lahko rezervoar genov za β-laktamaze.

Raziskava je bila izvedena v okviru znanstvenega projekta No.C-26/792.

Ključne besede: *E. coli*, APEC, odpornost proti antibiotikom

ABSTRACT

Avian pathogenic *Escherichia coli* (APEC) is the leading cause of avian colibacillosis globally and due to high morbidity and mortality rates poses a major economic problem for the poultry industry. Antibacterial drugs play an important role in the control of *E. coli* infections, but continuous use lead to the emergence of drug-resistant including multidrug-resistant strains. The aim of our study was to determine the prevalence of selected antibiotic resistance genes among 28 APEC strains isolated in the period 2016–2018 from various organs of chickens with colibacillosis. Genes encoding β-lactamases ($bla_{TEM\text{-},1}$, bla_{SHV}, $bla_{CTX\text{-}M}$, $bla_{OXA\text{-}1}$, $bla_{CMY\text{-}1}$), aminoglycoside 2'-N-acetyltransferase (*aacC2*) and region of class 1 integrons (*intI*) were studied. Specific PCR amplification of $bla_{TEM\text{-}1}$ gene was detected in 20 (71.4%) strains, the second most frequent was $bla_{CTX\text{-}M}$ (53.6%), while bla_{SHV} gene was detected only in one strain. Genes $bla_{CMY\text{-}1}$ and $bla_{OXA\text{-}1}$ were not found among studied strains. In 8 strains (28.6%) *aac2* gene was detected. The $bla_{TEM\text{-}1}$ + $bla_{CTX\text{-}M}$ combination was detected in 13 strains (46.4%), and the $bla_{TEM\text{-}1}$ + $bla_{CTX\text{-}M}$ + *aacC2* combination in 6 strains (21.4%). In 8 strains (28.6%) fragments of class 1 integrons (size of fragments varied from 800 to 2000 bp) were detected and two of these were located on conjugative plasmids. All amplified class 1 integrons were associated with the presence of ampicillin/cephalosporin resistance genes and only in two cases

were associated with aminoglycoside resistance gene. Thus, our data show that APEC strains are reservoirs of β-lactamase genes.

The study was carried out in the framework of the scientific project No.C-26/792.

Key words: *E. coli*, APEC, antibiotic resistance

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P16

NGS SEQUENCING AND BIOINFORMATIC ANALYSIS REVEALS MICRO RNAs IN PATHOGENIC PLANT FUNGI *Verticillium nonalfalfae*

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POVZETEK

Interferenca RNA je evolucijsko ohranjeni evkariontski regulatorni mehanizem, ki preko kratkih molekul RNA, kot so mikro molekule RNA (miRNA) oz. mikro RNA podobnim molekule (milRNA), uravnava izražanje genov na post-transkripcijskem nivoju. Nedavno je bilo dokazano, da se molekule milRNA izražajo tudi v filamentnih glivah, vendar njihova prisotnost še ni bila potrjena v patogeni glivi *Verticillium nonalfalfae*, ki povzroča simptome uvelosti in bolezni prevodnega sistema številnih kmetijsko pomembnih rastlin. Dva patotipa glive *V. nonalfalfae*, manj virulenti ter visoko virulentni, sta bila izolirana iz slovenskih nasadov hmelja, kjer je visoko virulentni patotip povzročil hude simptome uvelosti in odmiranje hmeljevih sadik. Iz obeh patotipov, manj virulentnega REC ter visoko virulentnega T2, ki smo jih pridobili iz Zbirke škodljivih organizmov Inštituta za hmeljarstvo in pivovarstvo Slovenije, smo izolirali frakcije molekul RNA, krajših od 200 nukleotidov. Določili smo nukleotidna zaporedja kratkih RNA z uporabo visoko zmogljivega sistema Ion Torrent ter napovedali glivne prekurzorje milRNA z uporabo programskega paketa MIReNA. Rezultate smo nadaljnje ročno pregledali na podlagi kriterijev za izbor rastlinskih kandidatnih prekurzorjev milRNA (Mishra et. al, 2015). Kandidatne prekurzorje smo validirali z metodo stem-loop RT-qPCR z uporabo milRNA specifičnih začetnih oligonukleotidov in eksperimentalno potrdili 10 novih milRNA v glivi *V. nonalfalfae*. S programom psRNATarget smo identificirali tarčne mRNA transkripte v genomu glive in trenutno z metodo 5' RLM-RACE potrjujemo interakcije milRNA s tarčnimi transkripti.

Ključne besede: *V. nonalfalfae*, milRNA, MIReNA, stem-loop RT-qPCR

ABSTRACT

RNA interference is an evolutionary conserved eukaryotic regulatory mechanism mediating gene expression post-transcriptionally trough small RNA molecules, including microRNA-like small RNAs (milRNAs), recently shown to exist in filamentous fungi. However, to date no milRNAs have been reported in the pathogenic plant fungi *Verticillium nonalfalfae*, a soil borne plant pathogen causing vascular wilt in many important crops worldwide. Two pathotypes of *V. nonalfalfae*, less virulent and highly virulent, have been isolated from Slovenian hop fields, where the highly virulent strain caused severe wilting symptoms and dieback of hop plants. Small RNAs from less virulent REC and highly virulent T2 isolates, provided by the fungal genebank of the Slovenian Institute for Hop Research and Brewing, were isolated and sequenced using high throughput NGS system Ion Torrent and fungal milRNA precursors predicted using MIReNA software. The results were further validated and milRNA precursor candidates selected manually using criteria for plant miRNA candidates proposed by Mishra et al. (2015). Candidate precursors were validated by stem-loop RT-qPCR using

milRNA specific primers and a total of 10 novel milRNAs were experimentally confirmed. With psRNATarget software the target mRNA transcripts were predicted in the fungal genome and the interactions are currently being validated using modified 5' RLM-RACE protocol

Key words: *V. nonalfalfae*, milRNA, MIReNA, stem-loop RT-qPCR

POSTERS – INTERACTION GENOME-ENVIRONMENT

Urška Skamen

P17: CONJUGAL TRANSFER FREQUENCY OF PLASMID pKJK5 BETWEEN *Escherichia coli* ISOLATES: INFLUENCE OF TEMPERATURE AND PRESENCE OF NUTRIENTS

Bernarda Strojin

P18: FREQUENCY OF CONJUGAL TRANSFER OF THE PLASMID pOX38 AND PLASMID pOX38a

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P17

CONJUGAL TRANSFER FREQUENCY OF PLASMID pKJK5 BETWEEN *Escherichia coli* ISOLATES: INFLUENCE OF TEMPERATURE AND PRESENCE OF NUTRIENTS

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POVZETEK

Escherichia coli (*E. coli*) je bakterija, ki je del normalne črevesne mikrobiote ljudi in živali s stalno telesno temperaturo. Iz črevesja se *E. coli* z iztrebki izloča v naravo, kjer se mikroorganizmi zaradi selekcijskega pritiska prilagajajo na spreminjajoče se okolje, vključno na prisotnost antibiotikov. To vodi k hitremu širjenju genov za antibiotske rezistence. Da bi lahko ocenili frekvenco konjugativnega prenosa plazmida pKJK5 med sevi *E. coli* v naravi, smo kot medij za konjugacijo uporabili rečni sediment. Poskuse konjugacije smo izvedli pri različnih temperaturah ter ob prisotnosti oziroma odsotnosti dodanih hranil. Izmerjene frekvence konjugacij smo primerjali s frekvencami konjugacij izmerjenimi na trdnem gojišču z in brez hranil, vzporedno pri istih temperaturah kot eksperiment v rečnem sedimentu. Tako smo lahko ugotavljali ne le vpliv temperature in prisotnosti hranil na frekvenco konjugacije, temveč tudi vpliv konjugacijskega medija. Ugotovili smo, da je frekvenca konjugacije tako v rečnem sedimentu kot na trdnem gojišču najvišja pri 34 °C, da pada s temperaturo ter je pri nekaterih temperaturah odvisna od prisotnosti hranil. Frekvenca konjugacije v rečnem sedimentu je pri vseh temperaturah nižja kot na trdnem gojišču, vendar pri višjih temperaturah še vedno znatna. Da povzamemo, rezultati kažejo, da je konjugacija plazmida pKJK5 med sevi *E. coli* v naravi možna, vendar v določenih razmerah.

Ključne besede: *E. coli*, konjugacija, plazmid, antibiotska rezistenca

ABSTRACT

Escherichia coli (*E. coli*) is a bacterium that is part of the gut microbiota of humans and other warm-blooded animals. With faeces *E. coli* is excreted into the natural environment, where microorganisms due to the selection pressure adapt to the changing environment, including the presence of antibiotics. This leads to quick spread of antibiotic resistance genes. To estimate the conjugal transfer frequency of plasmid pKJK5 between *E. coli* isolates in the natural environment, in conjugation experiments the river sediment was used as the medium for conjugal transfer. The conjugation assays were performed at different temperatures and in the presence and absence of nutrients. The obtained conjugal transfer frequencies were compared to conjugal frequencies determined in conjugation assays on solid medium with and without nutrients and at the same temperatures as in the river sediment medium. Thus, we were able to determine not only the influence of temperature and the presence of nutrients on conjugal transfer, but also the influence of conjugation medium. Our results showed that the conjugation frequency in river sediment, as well as on solid medium was highest at 34 °C and decreased with temperature and depended on the presence of nutrients at certain temperatures. The conjugation frequency was lower in the river

sediment at all temperatures but was still significant at higher temperatures. To conclude, based on the obtained results it can be assumed that pKJK5 conjugation between *E. coli* strains in natural environment is possible, however under certain conditions.

Key words: *E. coli*, conjugation, plasmid, antibiotic resistance

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P18

FREQUENCY OF CONJUGAL TRANSFER OF THE PLASMID pOX38 AND PLASMID pOX38a

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IZVLEČEK

Proti antibiotikom večkratno odporne bakterije postajajo čedalje večji problem. Geni za odpornost proti antibiotikom se pogosto nahajajo na konjugativnih plazmidih, ki so sposobni horizontalnega prenosa (konjugacija), kar je eden izmed razlogov za hitro širjenje genov odpornosti proti antibiotikom v bakterijski populaciji. Svetovna zdravstvena organizacija je leta 2017 objavila seznam bakterijskih skupin, za katere so nujno potrebne raziskave in razvoj novih antibiotikov. Osredotočili so se predvsem na po Gramu negativne bakterije, ki so odporne proti več različnim antibiotikom. Alternativa antibiotikom bi lahko postali bakteriocini, še posebej, če bi bili zapisi za njihovo sintezo na konjugativnih plazmidih, ki bi bili del na konjugaciji temelječih protimikrobnih dejavnikov. Na konjugaciji temelječi protimikrobni dejavniki, so sestavljeni namreč iz gena, ki je zapisan na konjugativnem plazmidu in kodira toksičen produkt, in gena, ki je zapisan na kromosomu in kodira imunost proti toksičnemu produktu. Konjugativni plazmid prenese gen v tarčno celico, kjer se izrazi in povzroči propad tarčne celice. Starčič Erjavec in sod. so konstruirali na konjugaciji temelječ protimikrobni dejavnik z genom za kolicin E7 na konjugativnem plazmidu in genom za imunost proti kolicinu E7 na kromosomu. Gen za kolicin E7 kodira DNazo, ki povzroči razgradnjo DNA in posledično propad recipientske celice. Določali smo frekvenco konjugacijskega prenosa plazmida z in brez zapisa za kolicin E7 v izbran recipientski sev ter preverjali vpliv minimalnega gojišča in vpliv prenašanja bakterij na frekvenco konjugacije. Frekvenca konjugacije je bila v LB po 24 urah $(7.6 \pm 1.2) \times 10^{-1}$. Ugotovili smo, da minimalno gojišče in prenašanje bakterij pri 4 °C na frekvenco konjugacije ne vplivata.

Ključne besede: *Escherichia coli*, konjugacija, konjugativni plazmidi, kolicin E7

ABSTRACT

Multidrug resistant bacteria are becoming an increasing problem. Often antibiotic resistance genes are carried on conjugative plasmids, plasmids that are capable of horizontal gene transfer (conjugation), which is a significant cause of the rapid spread of antibiotic resistance genes in bacterial populations. In 2017, the World health organization published a list of bacteria for which research and development of new antibiotics are urgently needed. They focused mainly on Gram-negative bacteria that are resistant to several different antibiotics. Bacteriocins could become an alternative to antibiotics, especially if genes for the bacteriocin synthesis would be carried on conjugative plasmids incorporated into conjugation-based antimicrobial systems. Such systems consist of two genes, the activity gene on a conjugative plasmid and the chromosomally encoded immunity gene. The conjugative plasmid transfers the activity gene into the target cell, where it is expressed and the target cell killed. Starčič Erjavec et al. constructed a conjugation-based antimicrobial system with the gene for colicin E7 and the E7 immunity gene. The colicin E7 gene

encodes a DNase that degrades the DNA, which results in the death of the target cell. We determined the conjugal transfer frequency of the plasmid with and without the colicin E7 gene into the selected recipient and the influence of minimal medium and bacterial convey at 4 °C on conjugation frequency. The frequency of conjugation of pOX38 in LB after 24 h was $(7.6 \pm 1.2) \times 10^{-1}$. Our results showed that neither minimal medium nor the bacterial transport affected the conjugation frequency.

Key words: *Escherichia coli*, conjugation, conjugative plasmids, colicin E7

INTRODUCTION

Multidrug resistant bacteria are becoming an increasing problem in the world. There are many causes or reasons connected with the increase in antibiotic resistance, among them the horizontal gene transfer. Several types of horizontal gene transfer exist, however with the rapid spread of antimicrobial resistance in bacterial population, most often conjugation, the gene transfer between two bacteria, which are in direct contact, is connected. In 2017, World health organization published a list of bacteria for which new antibiotics are urgently needed. The list was drawn up to promote research and development of new antibiotics.[1] Bacteriocins, toxins synthesized by bacteria, could become an alternative to antibiotics. Some bacteriocins are already in widespread use, for example the bacteriocin nisin, which is since 1953 used as a preservative in food industry and as a prophylactic in veterinary healthcare. Lactic acid bacteria often produce bacteriocins that allow the growth of useful bacteria by destroying useless ones.[2] Bacteriocin genes can enter into other bacterial cells with horizontal gene transfer (conjugation).[3]

Petkovšek et al. (2015) developed a bacteriocin - conjugation-based “kill”–“anti-kill” antimicrobial system (''kill'' – ''anti-kill'' antimicrobial system N4i pOX38a). This system has two genes, the bacteriocin (colicin E7) activity gene (“kill” gene), carried on a conjugative plasmid and the chromosomally encoded bacteriocin (colicin E7) immunity gene (“anti-kill” gene). By conjugative plasmid the “kill” gene is transferred into a recipient cell, where it is expressed and the recipient killed, namely the colicin E7 gene encodes a DNase that degrades the recipient’s DNA.[4]

The aim of this study was to determine the transfer frequency of the conjugative plasmid with and without the colicin E7 gene. For the donor strain the MG1655Ch pUC19i pOX38a and as the recipient the DH5α strain were used. We were also interested in the influence of minimal medium and bacterial transport at 4 °C on conjugation frequency.

MATERIAL AND METHODS

Bacterial strains and growth conditions. *E. coli* strains used in this study are listed in Table 1. All strains were grown in liquid LB medium with the appropriate antibiotics at 37 °C with aeration (180 rpm).

Table 1: Bacterial strains used in this study

Strain	Genotype and phenotype	Source
DH5α	*thi-1 hsdR17 gyrA96 recA1 endA1 glnV44 relA1 Φ80dlacZΔM15 ΔlacU169 phoA8*	NCCB (strain PC2955)
MG1655Ch pKJK5	*lacI-pLpp-mCherry*-Km^r pKJK5*::Plac::gfp*	Klümper et al., 2015
N4i pOX38a (ŽP)	Nissle 1917 (EcN) *immE7* Gm^r (N4i) with the plasmid pOX38:Cm with *colE7* activity gene	Petkovšek et al., 2012
MC4100 pUC19i	pUC19 with the *colE7* immunity gene Ap^r	Petkovšek et al., 2012
MRU1	N4i with plasmid pOX38:Cm without the *colE7*	Rumpret, 2014
BZB2110	*E. coli* with the plasmid pColE7-K317	NCCB (strain PC4209)
MG1655Ch	*lacI-pLpp-mCherry*-Km^r	This study
MG1655Ch pUC19i	*lacI-pLpp-mCherry*-Km^r with the plasmid pUC19 with the *colE7* immunity gene Ap^r	This study
MG1655Ch pUC19i pOX38	*lacI-pLpp-mCherry*-Km^r with the plasmids pUC19 with the *colE7* immunity gene Ap^r and pOX38	This study
MG1655Ch pUC19i pOX38a	*lacI-pLpp-mCherry*-Km^r with the plasmids pUC19 with the *colE7* immunity gene Ap^r and pOX38:Cm with *colE7* activity gene	This study

Conjugation in liquid medium. Overnight cultures of bacterial donor (MG1655Ch pUC19i pOX38a/pOX38) and recipient (DH5α) strains were diluted in a 1:100 ratio in 5 mL of fresh liquid LB medium without antibiotics and incubated at 37 °C with aeration (180 rpm) for 2 hours. Subsequently, 750 µL of the donor and recipient culture were joined for a conjugation mixture in a microcentrifuge tube and incubated at 37 °C for 1 h or 24 h. After the incubation, tenfold serial dilutions were prepared with 0.9 % NaCl and plated on selective LB plates for CFU counts of the transconjugants, donor and the recipient strain. The conjugation frequency was calculated using the following formula: conjugal transfer frequency = (CFU transconjugants) / (CFU recipient strain).

When conjugal transfer was performed in minimal medium M9, recipient and donor cultures, after the initial 2 hours of incubation of diluted overnight cultures in fresh medium, were centrifuged at 4000 rpm for 10 min. The supernatant was discarded and the harvested cells were resuspended in minimal medium M9. Centrifugation and resuspension was repeated twice. Then the donor and recipient cultures, in ratio 1:1 were joined for a conjugation mixture in a microcentrifuge tube and incubated at 37 °C for 1 h or 24 h. After the incubation, serial dilutions and plating on selective plates was performed as for the mating assays in LB medium.

The effect of bacterial transport at 4°C was determined by dividing the donor and recipient into two parallels before joining the strains in a conjugation mixture. One parallel was incubated at room temperature for half an hour. The other one was taken on a half an hour walk at 4 °C. Subsequently, the donor and recipient cultures, in a 1:1 ratio were joined in a conjugation mixture in

a microcentrifuge tube and incubated at 37 °C for 1 h. Subsequently, serial dilutions and plating on selective plates was performed as for the mating assays in LB medium.

All experiments were repeated three times and conjugation frequencies were calculated.

Loss of pKJK5 plasmid. A single colony of strain MG1655Ch pKJK5 was streaked on an LB plate with kanamycin and incubated at 42 °C for 2 days and then incubated at 4 °C for 3 days. Afterwards, only the outer edge of the colony was streaked on a new LB plate with kanamycin and incubated at 42 °C overnight. The next day, colonies were streaked on LB plate with trimethoprim and incubated at 37 °C overnight. The colony, which did not grow, had lost the plasmid. As a control, the same colony was streaked on LB plate with kanamycin and trimethoprim. The cells which had lost the plasmid grew into colonies on LB plate with only kanamycin.

Preparation of strain MG1655Ch pUC19i pOX38a/pOX38 (isolation of plasmid pUC19i, preparation of competent cells, transformation of competent cells, conjugation on solid medium). First of all, plasmid pUC19i was isolated from strain MC4100 pUC19i using »GeneJet Plasmid Miniprep Kit« following the manufacturer's instructions. Before transformation of pUC19i into MG1655Ch, the MG1655Ch competent cells were prepared. A single colony of MG1655Ch strain was inoculated in 5 mL of LB with the appropriate antibiotic and incubated at 37 °C and 180 rpm overnight. Overnight cultures was inoculated in ratio 1:100 into LB without antibiotic and incubated at 37 °C and 180 rpm to OD_{600} of 0.43. 34 mL were transferred to a 50-mL centrifuge tube and incubated on ice for 10 min. Cells were harvested by centrifugation at 4000 rpm for 10 min. Supernatant was discarded and cells were resuspended in 7 mL of ice cold 0.1 M $CaCl_2$. 100 µL of competent cells were mixed with 1 µL of plasmid pUC19i DNA. The mixture was incubated on ice for 30 min. Afterwards, cells were incubated at 42 °C in a water bath for 90 s and then immediately transferred on ice for 2 min. 400 µL of LB were added to the mixture and incubated at 37 °C and 180 rpm for 1 h. 100 µL of the mixture were plated on an LB plate with ampicillin and kanamycin. LB plate was incubated at 37 °C overnight. In order to transfer the conjugative plasmid into strain MG1655 pUC19i, single colonies of strain N4i pOX38a/MRU1 and MG1655Ch pUC19i were streaked one over the other on a LB plate and incubated at 37 °C overnight. After incubation, the mixture was streaked on a LB plate with appropriate antibiotics for the selection of MG1655Ch pUC19i pOX38a/pOX38.

RESULTS AND DISCUSSION

In order to determine the conjugal transfer frequencies of the two plasmids pOX38 and pOX38a into the laboratory recipient strain DH5α mating assays were performed in liquid LB medium and liquid M9 minimal medium. The obtained results are shown in Table 2 and Table 3. The conjugal transfer frequency of pOX38 was expected to be higher than the conjugal frequency of pOX38a, because the pOX38a plasmid carries the colicin toxic activity gene. But, some transconjugants still grew on selective LB plates. For such colonies further experiments to determine the origin of the colony (mutated donor/recipient or real transconjugants) would be needed.

Table 2: Conjugal frequencies of donor strain MG1655 pUC19i pOX38 and recipient strain DH5α in LB and M9 medium. Experiments were performed in triplicates. Standard error is used as an indicator of variation.

	MG1655Ch pUC19i pOX38 × DH5α					
	LB			**M9**		
	Before conjugation	After 1 hour	After 24 hours	Before conjugation	After 1 hour	After 24 hours
Number of recipient strain cells (CFU)	$(1.8±1.8)*10^7$	$(8.7±5.1)*10^6$	$(4.3±1.7)*10^7$	$(1.2±1.3)*10^7$	$(5.4±4.1)*10^6$	$(9.6±6.1)*10^6$
Number of donor strain cells (CFU)	$(2.3±1.4)*10^7$	$(2.2±1.6)*10^7$	$(2.2±1.6)*10^8$	$(2.5±3.9)*10^7$	$(2.6±3.9)*10^7$	$(2.1±2.6)*10^7$
Number of transconjugants (CFU)	0	$(3.1±4.5)*10^5$	$(3.3±1.3)*10^7$	0	$(2.6±4.4)*10^5$	$(5.5±6.0)*10^5$
Conjugal frequency	**0**	**$(0.9±1.5)*10^{-1}$**	**$(7.6±1.2)*10^{-1}$**	**0**	**$(2.5±4.4)*10^{-1}$**	**$(1.6±2.5)*10^{-1}$**

Frequencies of conjugal transfer of plasmid pOX38 in minimal medium M9 were approximately equal as in liquid LB. This result was unexpected, as lack of nutrients on the M9 medium should influence the conjugal frequency. As expected, no transconjugants were detected in minimal medium M9 with plasmid pOX38a.

Table 3: Conjugal frequencies of donor strain MG1655 pUC19i pOX38a and recipient strain DH5α in LB and M9 medium. Experiments were performed in triplicates. Standard error is used as an indicator of variation.

	MG1655Ch pUC19i pOX38a × DH5α					
	LB			**M9**		
	Before conjugation	After 1 hour	After 24 hours	Before conjugation	After 1 hour	After 24 hours
Number of recipient strain cells (CFU)	$(2.5±2.1)*10^7$	$(2.4±1.9)*10^6$	$(6.9±5.8)*10^6$	$(1.7±1.8)*10^6$	$(5.0±1.7)*10^6$	$(1.4±0.4)*10^6$
Number of donor strain cells (CFU)	$(3.7±2.1)*10^7$	$(5.2±3.4)*10^7$	$(4.3±1.2)*10^8$	$(7.9±3.4)*10^6$	$(5.4±1.7)*10^6$	$(5.5±0.5)*10^6$
Number of transconjugants (CFU)	0	$(2.1±2.1)*10^2$	$(5.3±5.5)*10^5$	0	0	0
Conjugal frequency	**0**	**$(5.7±5.2)*10^{-5}$**	**$(6.1±6.9)*10^{-2}$**	**0**	**0**	**0**

We found that the 30-min transport at 4 °C with donor (MG1655Ch pUC19i pOX38) and recipient (DH5α) strains before conjugation did not affect the conjugal transfer frequencies (Table 4). Frequency of conjugation was the same with or without the bacterial transport. That means that incubation at 4 °C and transport of donor and recipient strains before conjugation does not affect conjugal frequencies.

Table 4: Conjugal frequencies of donor strain MG1655 pUC19i pOX38 and recipient strain DH5α. Experiments were performed in triplicates. Standard error is used as an indicator of variation.

	With convey		**Without convey**	
	Before conjugation	After 1 hour	Before conjugation	After 1 hour
Number of recipient strain cells (CFU)	$(1.1\pm1.0)*10^6$	$(1.0\pm0.7)*10^6$	$(2.1\pm1.3)*10^6$	$(1.8\pm1.5)*10^6$
Number of donor strain cells (CFU)	$(1.4\pm0.7)*10^7$	$(3.1\pm3.1)*10^7$	$(1.9\pm1.3)*10^7$	$(4.0\pm3.1)*10^7$
Number of transconjugants (CFU)	0	$(8.7\pm8.1)*10^4$	0	$(1.7\pm1.9)*10^5$
Conjugal frequency	**0**	$\mathbf{(7.9\pm2.9)*10^{-2}}$	**0**	$\mathbf{(8.0\pm3.2)*10^{-2}}$

To conclude, pOX38 plasmid conjugative transfer frequencies were relatively high, while the pOX38a conjugative transfer frequencies were as expected lower. To our surprise, neither minimal medium nor bacterial transport and incubation at 4 °C affected the conjugation frequency. For future experiments we plan to test the effect of different temperatures and different ratios of donor and recipient cells joined in a conjugation mixture.

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