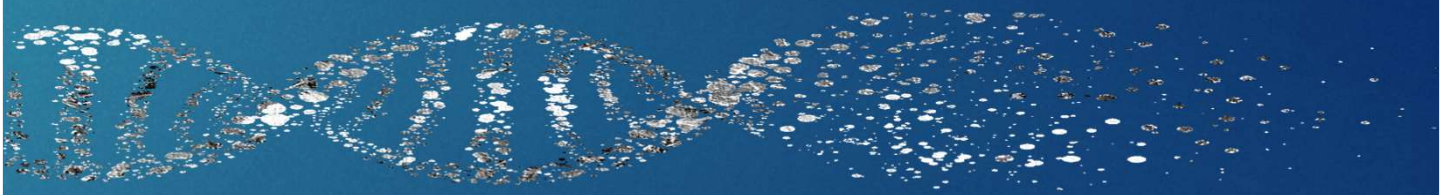
SGD2020

8th Colloquium on Genetics

Organised by Genetic Society of Slovenia (GSS)
and Slovenian Society of Human Genetics (SSHG)

Book of Abstracts



28th September 2020 | online event

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BOOK OF ABSTRACTS

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Gregor GORJANC, *University of Edinburgh, Roslin Institute*

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	9:40 – 10:10	**Gregor GORJANC,** *University of Edinburgh, Roslin Institute*
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Poster session 1	10:50 – 10:55	**Expression of Long Non-Coding RNA *CCAT1* in cancers of the larynx, pharynx and oral cavity** Davor BABIČ, Bogdan ČIZMAREVIĆ, Tomaž BÜDEFELD, Uroš POTOČNIK
	10:55-11:00	**The microenvironment of the tumour is altered after gene electrotransfer of plasmid DNA encoding the proinflammatory chemokines CCL5 and CCL17** Tim BOŽIČ, Boštjan MARKELC, Simona KRANJC BREZAR, Gregor SERŠA, Maja ČEMAŽAR
	11:00-11:05	**Epigenetic regulation of the *MIR137/MIR2682* locus in Head and neck squamous cell carcinoma** Helena ČELEŠNIK, Tomaž BÜDEFELD, Bogdan ČIZMAREVIĆ, Uroš POTOČNIK
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Abstracts | Izvlečki

The Past, Present and Future of Quantitative Genetics

Preteklost, sedanjost in prihodnost kvantitativne genetike

Gregor GORJANC[1,2]

[1]*The Roslin Institute, University of Edinburgh*
[2]*Biotechnical Faculty, University of Ljubljana*

ABSTRACT

This presentation highlights four eras of quantitative genetics. **The origin**. Quantitative genetics originates from statistical analyses of human phenotypic variation. These analyses showed that the phenotypes of relatives correlate and that the magnitude of correlation varies predictably between different groups of relatives. These were the early days of genetics - Mendel's laws were rediscovered and the Mendelian view of inheritance for individual genes was debated against the biometric view of observed continuous variation. Fisher settled the debate by showing equivalence between the views for traits influenced by a large number of genes. He showed this with a linear approximation of phenotype with a sum of additive and non-additive genetic values and environmental deviation. **The application and a perceived demise.** Statisticians soon developed flexible models and efficient methods for applications of quantitative genetics. Uptake in animal and plant breeding was particularly successful. Crucial components for the success were the genetic and statistic foundations as well as adequate data structures with phenotyped genotypes, or their close relatives, in different environments. The applications flourished and drove most of the genetic gains. However, molecular genetics overtook the attention and funding with the promise of actually finding and working with genes. **The genomic revival**. Molecular genetics kick-started a continuous avalanche of genome-wide polymorphism data and enabled an upgrade of quantitative genetics. The upgrade replaced the expected pedigree correlations with realised genomic correlations. Applications followed immediately in the form of genome-wide association studies for gene hunting and genomic predictions of additive and non-additive genetic values for ranking individuals. Genomics increased the rate of genetic gain in agriculture and brought the promise of personalised medicine. **The outlook.** While genome-wide polymorphism data upgraded quantitative genetics, there are many unresolved problems. Genomic predictions are at best population-specific, and we mostly still do not understand the intricacies of phenotypic variation. However, recent trends in large scale whole-genome sequencing, phenotype data capture, data science and genome editing promise exciting future of quantitative genetics.

IZVLEČEK

Predstavitev obravnava štiri obdobja kavntitativne genetike. **Izvor.** Kvantitativna genetika izhaja iz statističnih analiz fenotipske variabilnosti pri človeku. Te analize so pokazale, da so fenotipi sorodnikov korelirani in da se stopnja korelacije med različnimi skupinami sorodnikov predvidljivo razlikuje. To so bili zgodnji časi genetike - Mendelovi zakoni so bili znova odkriti in Mendelski pogled na dedovanje posameznih genov se je

razpravljal v primerjavi z biometričnim pogledom na opaženo zvezno variabilnost. Fisher je razpravo razrešil s prikazom enakovrednosti pogledov za lastnosti, ki so pod vplivom velikega števila genov. To je pokazal z linearnim približkom fenotipa z vsoto aditivnih in neaditivnih genetskih vrednosti in odklonom zaradi okolja. **Uporaba in navidezen zaton.** Statistiki so kmalu razvili prilagodljive modele in učinkovite metode za uporabo kvantitativne genetike. Še posebej uspešen je bil prevzem pri selekciji živali in rastlin. Ključnega pomena za uspeh so bile genetske in statistične osnove ter ustrezne podatkovne strukture s fenotipiziranimi genotipi, ali njihovimi bližnjimi sorodniki, v različnih okoljih. Aplikacije so bile uspešne in zaslužne za večino genetskega napredka. Kljub temu je molekularna genetika prevzela pozornost in financiranje z obljubo, da bo dejansko našla in delala z geni. **Genomska oživitev.** Molekularna genetika je sprožila neprekinjen plaz podatkov o genomskih polimorfizmih in omogočila nadgradnjo kvantitativne genetike. Nadgradnja je pričakovane rodovniške korelacije nadomestila z realiziranimi genomskimi korelacijami. Aplikacije so takoj sledile v obliki genomskih asociacijskih študij za lov na gene in genomskih napovedi aditivnih in neaditivnih genetskih vrednosti za razvrščanje posameznikov. Genomika je povečala stopnjo genetskega napredka v kmetijstvu in prinesla obljubo o posamezniku prilagojenem zdravljenju. **Obeti.** Čeprav so podatki o genomskih polimorfizmih nadgradili kvantitativno genetiko, obstaja veliko nerešenih problemov. Genomske napovedi so v najboljšem primeru specifične za dano populacijo in večinoma še vedno ne razumemo zapletenosti fenotipske variabilnosti. Kljub temu nedavni trendi v odčitavanju celotnega genoma, zajemanju fenotipskih podatkov, znanosti o delu s podatki in urejanju genoma obljubljajo vznemirljivo prihodnost kvantitativne genetike.

Expression of Long Non-Coding RNA *CCAT1* in Cancers of the Larynx, Pharynx and Oral Cavity

Izražanje dolge nekodirajoče RNA *CCAT1* pri rakih grla, žrela in ustne votline

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ABSTRACT

Head and Neck Cancer (HNC) is a heterogeneous group of cancers that includes cancers of the lips, oral cavity, salivary glands, nasal cavity, paranasal sinuses, pharynx, larynx and head and neck lymph nodes. Previous studies have shown increased expression of long non-coding RNA (ncRNA) *CCAT1* (CCAT1) in HNC and its role in cell proliferation, clonality, migration and invasion; however, the exact role of *CCAT1* in HNC carcinogenesis is unknown. The aim of the present study was to evaluate the expression of *CCAT1* in cancers of the larynx, pharynx and oral cavity with clinicopathological correlations. *CCAT1* expression was examined using RT-PCR on paired tumour-normal tissue samples from 34 patients with HNC, and clinical and pathological data were collected from the respective medical files. *CCAT1* ncRNA levels differed between tumours of the larynx, pharynx and oral cavity and normal tissue at stage cT2, but not other cT stages, with tumours having higher ($p<0.05$) *CCAT1* expression than normal tissue. Tumours classified by the cN, pT and pN stages did not show any differences in *CCAT1* expression compared to normal tissue. Similarly, the tumour location and invasion, as well as risk factors including tobacco smoking, alcohol consumption and HPV16 infection, were not associated with *CCAT1* expression. Our data are in line with previous observations suggesting that tobacco smoking, alcohol consumption and HPV16 infection are not associated with *CCAT1* expression in HNC. The clinical significance of higher *CCAT1* expression in stage cT2 head and neck tumours is currently unknown and requires further examination.

***Key words**: head and neck cancer, lncRNA, CCAT1, invasion, RT-PCR*

IZVLEČEK

Rak glave in vratu (HNC) je heterogena skupina rakov, ki vključuje rak ustnic, ustne votline, žlez slinavk, nosne in obnosne votline, žrela, grla in vratnih bezgavk. Predhodne študije so pokazale povečano izražanje dolge nekodirajoče RNA (ncRNA) *CCAT1* (*CCAT1*) pri HNC in njeno vlogo pri celični proliferaciji, klonalnosti, migraciji in invaziji; a vendar

natančna vloga *CCAT1* pri kancerogenezi HNC ostaja nepoznana. Cilj študije je bil s klinično-patološkimi korelacijami proučiti izražanje *CCAT1* pri rakih grla, žrela in ustne votline. Na vzorcu 34 bolnikov je bilo z RT-PCR proučeno izražanje *CCAT1* v zdravem in tumorskem tkivu, klinični in patološki podatki pa so bili pridobljeni iz ustreznih zdravstvenih kartotek. Vrednosti ncRNA *CCAT1* so se razlikovale med tumorskim tkivom grla, žrela in ustne votline ter zdravim tkivom pri stopnji cT2 z višjim izražanjem *CCAT1* v tumorskem kot zdravem tkivu ($p<0,05$), razlik pa ni bilo pri drugih stopnjah cT. Tumorji razvrščeni po cN, pT in pN stadijih niso pokazali razlik v izražanju *CCAT1* v primerjavi z zdravim tkivom. Podobno lokacija tumorja in invazija, kot tudi dejavniki tveganja, vključno s kajenjem, uživanjem alkohola in okužbo s HPV16, niso bili povezani z izražanjem *CCAT1*. Naši podatki so v skladu s predhodnimi ugotovitvami in kažejo, da kajenje, uživanje alkohola in okužba s HPV16 niso povezani z izražanjem *CCAT1* pri HNC. Kliničen pomen povišanega izražanja *CCAT1* pri stadiju cT2 tumorja glave in vratu trenutno še ni poznan in zahteva nadaljnjo obravnavo.

***Ključne besede**: rak glave in vratu, lncRNA, CCAT1, invazija, RT-PCR*

The microenvironment of the tumour is altered after gene electrotransfer of plasmid DNA encoding the proinflammatory chemokines CCL5 and CCL17

Genski elektroprenos plazmidne DNA z zapisom za vnetna kemokina CCL5 in CCL17 spremeni tumorsko mikrookolje

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ABSTRACT

Proinflammatory chemokines mediated immune cell migration in combination with immune cell activating therapy such as irradiation is one of the potential modalities in cancer immunotherapy. Using plasmid DNA encoding either the proinflammatory chemokines CCL5 or CCL17, we investigated the effects of these two chemokines on cell viability, cytokine expression and tumour growth. The transfection of plasmid DNA was performed by lipofection *in vitro* and gene electrotransfer (GET) *in vivo*. The expression of cytokines was determined by qRT-PCR. The viability of murine breast (4T1, E0771) and colon (CT26, MC38) cancer cells remained above 80 % 48 h after lipofection with plasmids encoding CCL5 or CCL17. Concurrent expression analysis of 11 cytokines 48 h after lipofection showed a significantly increased expression of CCL5, CCL17 and moderately elevated levels of IL-6 and CXCL10 in the surviving cells. The use of GET after intratumoral injection of plasmids encoding either CCL5 or CCL17 in CT26 and 4T1 murine tumour model (animal license: U34401-1/2015/43) resulted in an average tumour growth delay of 4,6 days. The expression analysis of 7 cytokines after GET showed an increased expression of both chemokines, while the levels of IL-6, IL-12 and IFNγ were only slightly increased. An altered expression profile, both after lipofection and GET is typical for inflammation. GET of plasmids encoding CCL5 or CCL17 in combination with irradiation in the CT26 and 4T1 tumour model resulted in a significant delay in tumour growth and even in some mice without tumours. Based on the microenvironment of the inflamed tumour induced by our therapy, future experiments will therefore focus on elucidating the infiltration of immune cells into the treated tumours and determining the optimal time window for the combination of CCL5 and CCL17 GET with irradiation.

***Key words**: chemokines, CCL5, CCL17, gene electrotransfer, irradiation, cytokine expression profile*

IZVLEČEK

Migracija imunskih celic, posredovana s strani vnetnih kemokinov, v kombinaciji z obsevanjem, ki aktivira imunske celice, predstavlja enega od potencialnih pristopov v imunoterapiji raka. Z uporabo plazmidne DNA, z zapisom vnetnih kemokinov CCL5 ali CCL17 smo raziskovali vpliv teh dveh kemokinov na viabilnost celic, ekspresijo citokinov in rast tumorjev. Transfekcija plazmidne DNA je bila izvedena z uporabo lipofekcije *in vitro* in genskega elektroprenosa (GET) *in vivo*. Ekspresija citokinov je bila določena z metodo qRT-PCR. Stopnja preživetja mišjih celic raka dojke (4T1, E0771) in raka debelega črevesja (CT26, MC38) je bila 48 h po lipofekciji s plazmidoma, z zapisom za CCL5 ali CCL17, nad 80 %. Vzporedna analiza ekspresije 11 citokinov v preživelih celicah 48 h po lipofekciji je pokazala pomembno višje izražanje CCL5 in CCL17, ter rahlo povišano raven izražanja IL-6 in CXCL10. Aplikacija GET po intratumorskem injiciranju plazmidov z zapisom za CCL5 ali CCL17 v mišja tumorska modela CT26 in 4T1 vodi v povprečju do 4,6 dnevnega zaostanka v rasti tumorjev (dovoljenje: U34401-1/2015/43). Analiza ekspresije 7 citokinov po GET je pokazala povišano izražanje obeh kemokinov, med tem ko je bila raven izražanja IL-6, IL-12 in IFNγ le rahlo povišana. Spremenjeno izražanje tako po lipofekciji, kot tudi po GET, je tipično za vnetje. GET plazmidov z zapisom za CCL5 in CCL17 v kombinaciji z obsevanjem na tumorskem modelu CT26 in 4T1 vodi v značilen zaostanek v rasti tumorjev in celo miši pri katerih je tumor popolnoma izginil. Na podlagi vnetnega tumorskega mikrookolja, ki je posledica naše terapije, bodo prihodnji poskusi osredotočeni na preučevanje infiltracije imunskih celic v tretirane tumorje in k določevanju ustreznega časovnega okna za kombiniranje GET za kemokina CCL5 in CCL17 ter obsevanja.

***Ključne besede**: kemokini, CCL5, CCL17, genski elektroprenos, obsevanje, ekspresijski profil citokinov*

Epigenetic regulation of the *MIR137/MIR2682* locus in head and neck squamous cell carcinoma

Epigenetska regulacija lokusa *MIR137/MIR2682* pri ploščatoceličnem karcinomu glave in vratu

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ABSTRACT

Head and neck squamous cell carcinoma (HNSCC) is a type of malignancy that arises from the epithelial cells of the head and neck. It is one of the most common carcinomas worldwide, characterised by high aggressiveness and low survival. There is a need for effective diagnostic and prognostic HNSCC biomarkers. MicroRNAs, small non-coding RNAs, have been known to play important roles in the initiation and progression of various cancers, and their dysregulation is frequently seen in malignancies. MicroRNA miR-137 has been associated with various cancers, including HNSCC. This microRNA is located in the host gene *MIR137HG*. In the same host gene, separated by a CpG island, lies another microRNA, miR-2682. In this work, we set out to investigate the *MIR137/MIR2682* locus in HNSCC. Paired cancerous and adjacent noncancerous tissues were collected from eighty-eight HNSCC patients at University Medical Centre Maribor, and detailed clinical data was collected. MicroRNA expression in paired tissues was analysed by qRT-PCR and CpG methylation was determined by COBRA. Expression and methylation data were correlated with clinical data. The CpG island in the MIR137/MIR2682 locus was significantly hypermethylated ($p<0.01$) in cancerous tissues compared to adjacent healthy tissues. Both miR-137 and miR-2682 were downregulated ($p<0.01$) in HNSCC cancerous tissues, with oropharyngeal tumours showing the largest decrease in expression. Our data are in line with the previously proposed role of miR-137 as a tumour-suppressor and suggest that miR-2682 may also function as a tumour-suppressor.

***Key words**: HNSCC, head and neck cancer, miR-137, miR-2682, epigenetic, methylation*

IZVLEČEK

Ploščatocelični karcinom glave in vratu (HNSCC) je maligno obolenje, ki nastane iz epitelijskih celic v glavi ali vratu. Gre za enega najpogostejših karcinomov, za katerega je značilna visoka agresivnost in nizko preživetje,

zato se pojavlja vse večja potreba po učinkovitih diagnostičnih in prognostičnih biomarkerjih. Znano je, da imajo mikro RNA, majhne nekodirajoče RNA, pomembno vlogo pri iniciaciji in napredovanju različnih vrst raka. Njihovo spremenjeno izražanje pogosto opazimo pri različnih malignih boleznih. Mikro RNA miR-137 je bila opisana v povezavi z različnimi vrstami raka, vključno s HNSCC. MikroRNA-137 se nahaja v gostiteljskem genu MIR137HG, v katerem se nahaja še miR-2682, ločuje pa ju CpG-otoček. V našem delu smo raziskali lokus MIR137/MIR2682 pri bolnikih s HNSCC. Oseminosemdeset bolnikov s HNSCC je bilo vključenih v študijo. Na UKC Maribor so vsakemu bolniku odvzeli parne vzorce rakavega in zdravega okoliškega tkiva ter zbrali podrobne klinične podatke bolnika. V tkivih smo z metodo qRT-PCR analizirali izražanje mikro RNA, metilacijo CpG otočka pa smo določili z metodo COBRA. Podatke o izražanju in metilaciji smo korelirali s kliničnimi podatki. CpG-otoček v lokusu MIR137/ MIR2682 je kazal hipermetiliranost v rakavih tkivih v primerjavi s sosednjimi zdravimi tkivi ($p<0,01$). Tako miR-137 kot miR-2682 sta bili v rakavih tkivih znatno nižje izraženi ($p<0,01$), pri čemer so tumorji v orofarinksu pokazali največje znižanje. Naši rezultati se skladajo z objavljenimi podatki, ki miR-137 pripisujejo vlogo tumor-supresorja, ter nakazujejo podobno vlogo za miR-2682.

Ključne besede: *HNSCC, rak glave in vratu, miR-137, miR-2682, epigenetsko, metilacija*

Optimising the cultivation of head and neck cancer cells enables enrichment of stem-like cells

Optimizacija gojenja celic raka glave in vratu vodi do obogatitve z matičnim celicam podobnimi celicami

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ABSTRACT

Cancer stem cells (CSCs) represent a critical subset of cancer cells, known for their resistance to conventional therapies and preserving a tumour even after treatment destroyed/eliminated most of the tumour cells. Due to the need for CSC-targeted therapies and their CSC rarity in primary tissues, there is a growing need for a CSC-enrichment model, allowing CSC enrichment and thus the high-throughput screening (HTS) study of active ingredients with anti-CSC activity. Using head and neck squamous cell carcinoma cell line FaDu, round-bottom ultra-low adherent (ULA) microplates, and proper stem medium, enabled the development of head and neck cancer stem cell-enriched spheroid model (SCESM), suitable for HTS analyses of anti-CSC compounds. Optimised growth conditions, i.e. cell concentration, medium composition and cultivation length, enabled the formation of compact uniform spatially separated spheroids. When compared to the adherent cell line and multi-cellular tumour spheroid model (MCTS), a well-known HTS cell model, the SCESM showed an increased mRNA or protein expression of all CSC markers tested (CD44, CD73, CD90 and CD133). Additional confocal analysis confirmed the presence of fast proliferating cells only in the outer rim of the SCESM spheroids, with poorly/not proliferating cells deeper in. ATRA treatment strongly reduced the expression of selected stem markers and confirmed the sensitivity of SCESM. Growing cells in optimised 3-dimensional model enabled the development of spheroids with stem-like characteristics, while simple protocol, microplate format, high detection signal, and a set of CSC readout markers, make the developed model suitable for high-throughput anti-CSC compounds screening.

***Key words**: head and neck squamous cell carcinoma, cancer stem cells, 3D/spheroid culture/mode, high-throughput screening, anti-cancer stem cell compounds, ATRA, stem cell marker*

IZVLEČEK

Matične celice raka (MCR) predstavljajo kritično podskupino rakastih celic, s sposobnostjo samoobnavljanja in

diferenciacije ter ohranjanjem tumorja tudi po tem, ko zdravljenje uniči večino preostalih celic tumorja. Zaradi potrebe po terapijah ciljanih na MCR in njihove redkosti v primarnih tkivih je narasla potreba po modelu, ki bi omogočil obogatitev MCR ter hkrati dopuščal proučevanje učinkovin z aktivnostjo usmerjeno proti MCR z analizami visokozmogljivih presejalnih testov (VPT). Z uporabo celic ploščatoceličnega karcinoma glave in vratu FaDu, mikroploščic z ultra nizkim oprijemom (ULA) z okroglim dnom in matičnega medija smo razvili nov sferoidni model raka glave in vratu, obogaten z matičnimi celicami raka (SM), primeren za analize z VPT. Optimizirani rastni pogoji, tj. koncentracija celic, sestava medija in trajanje gojenja, so omogočili nastanek uniformih, prostorsko ločenih sferoidov. V primerjavi z adherentnimi celicami in modelom večceličnega tumorskega sferoida (MVTS), dobro znanim VPT celičnim modelom, je SM pokazal povečano mRNA in proteinsko izražanje markerjev matičnih celic (CD44, CD73, CD90 in CD133). Dodatna konfokalna analiza SM je potrdila prisotnost hitro proliferirajočih celic le na zunanjem robu sferoida, globje v sferoidu pa prisotnost mirujočih se celic. Tretiranje SM z ATRA je močno zmanjšalo izražanje izbranih markerjev matičnih celic in potrdilo občutljivost SM za proučevanje učinkovin. Gojenje celic v optimiziranem SM je omogočilo razvoj sferoidov z matičnimi značilnostmi, medtem ko preprost protokol, format v mikroploščah, močan signal za zaznavanje in nabor markerjev za zasledovanje matičnosti omogočajo uporabo SM za proučevanje anti-MCR spojin z analizami VPT.

***Ključne besede**: ploščatocelični karcinom glave in vratu, matične celice raka, 3D/sferoidna kultura/model, visokozmogljivi presejalni testi, učinkovine z delovanjem proti matičnim celicam raka, retinojska kislina, markerji matičnosti*

Different expression of DNA sensors and cytokines in response to ionising radiation in HPV negative and HPV positive oropharyngeal squamous cell carcinoma *in vitro*

Različno izražanje senzorjev DNA in citokinov kot odgovor na ionizirajoče sevanje pri HPV negativnemu in HPV pozitivnemu ploščatoceličnemu karcinomu ustnega žrela *in vitro*

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ABSTRACT

Human papillomavirus (HPV) infection is one of the major risk factors for oropharyngeal squamous cell carcinoma (OPSCC). Patients with OPSCC are primarily treated with radiotherapy and with or without chemotherapy. The DNA that accumulates in the cytosol of cells due to the damage induced by X-ray radiation can be sensed by specific pattern recognition receptors, i.e. DNA sensors. They can induce the production of various cytokines through several signalling pathways, which can then mediate the anti-tumour immune response and, consequently, impact the radiosensitivity of tumours. Clinical studies suggest that HPV positive OPSCC are more radiosensitive than HPV negative ones, however, the exact mechanisms are still not known. Therefore, the aim of our study was to investigate the involvement of DNA sensing pathways in response to radiation in HPV positive and HPV negative OPSCC *in vitro* and possible differences between them with respect to HPV viral load. HPV positive (2A3 and UPCI: SCC090) and HPV negative (FaDu and Detroit) OPSCC cell lines were irradiated with different single doses (0, 4 and 8 Gy). After 24 and 48 hours, respectively, RNA was isolated and reverse transcribed into cDNA. qRT-PCR was used to determine viral load (expression of E6 and E7 viral oncoprotein mRNAs), expression of DNA sensors and cytokines. Our results showed that the expression of DNA sensors (STING, RIG-I, DDX60, DAI and IFI 16) and cytokines (IFN β, TNF α and IL 1B) after irradiation was increased in HPV negative cell lines but not in HPV positive ones. In conclusion, the results indicate that on the *in vitro* level, DNA sensors are differentially expressed in response to irradiation in OPSCC cell lines based on HPV infection. Further *in vivo* studies are needed to elucidate the involvement of the immune system in the response.

Keywords: *human papillomavirus, oropharyngeal squamous cell carcinoma, irradiation, DNA sensors*

IZVLEČEK

Okužba s humanim papilomavirusom (HPV) je eden izmed glavnih dejavnikov tveganja za nastanek ploščatoceličnega karcinoma ustnega žrela (PCKO). Bolnike s PCKO primarno zdravimo samo z radioterapijo ali v kombinaciji s kemoterapijo. Ionizirajoče sevanje povzroči poškodbo celic, kar privede do kopičenja DNA v citosolu. Slednjo zaznajo specifični receptorji – senzorji DNA. Ti skozi več signalnih poti inducirajo nastajanje različnih citokinov, ki lahko nato izzovejo protitumorski imunski odziv in posledično vplivajo na radiosenzitivnost tumorjev. Klinične raziskave nakazujejo, da so HPV pozitivni PCKO bolj radiosenzitivni v primerjavi s HPV negativnimi, vendar mehanizmi še niso raziskani. Cilj naše študije je bil raziskati vpletenost poti zaznavanja senzorjev DNA *in vitro* pri HPV pozitivnih in HPV negativnih PCKO kot odgovor na obsevanje ter morebitne razlike med njimi glede na virusno breme. HPV pozitivne (2A3 in UPCI:SCC090) ter HPV negativne (FaDu in Detroit) PCKO celične linije smo obsevali z različnimi enkratnimi dozami (0, 4 in 8 Gy). Po 24 in 48 urah smo izolirali RNA ter kasneje napravili prepis v cDNA. Za določanje virusnega bremena (izražanje mRNA onkoproteinov E6 in E7) in izražanja različnih senzorjev DNA ter citokinov smo uporabili qRT-PCR. Naši rezultati so pokazali, da se je izražanje senzorjev DNA (STING, RIG-I, DDX60, DAI in IFI 16) in citokinov (IFN β, TNF α in IL 1B) po obsevanju pri HPV negativnih celičnih linijah povečalo, ne pa tudi pri HPV pozitivnih. Rezultati nakazujejo, da se na *in vitro* ravni senzorji DNA različno izražajo kot odziv na obsevanje v celičnih linijah PCKO z različnim HPV statusom. Nadaljnje študije *in vivo* so potrebne za razjasnitev vpletenosti imunskega sistema v odziv.

Ključne besede: *humani papilomavirus, ploščatocelični karcinom ustnega žrela, ionizirajoče sevanje, senzorji DNA*

Effective melanoma gene therapy with the combination of *Il2* and *Il12*

Učinkovita genska terapija z *Il2* in *Il12* za zdravljenje melanoma

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ABSTRACT

Immunotherapy has become an important approach for treating cancer. One example is the use of cytokines such as interleukin 2 (IL-2) and 12 (IL-12) that directly stimulate immune cells at the tumour site. The application of the aforementioned cytokines was primarily in the recombinant protein form, but nowadays, it is replaced by the gene therapy approach. The aim of our study was to determine the cytotoxic and antitumor effects of gene electrotransfer of plasmids encoding *Il-2* (pIL-2) and *Il-12* (pIL-12) to B16F10 melanoma cells and tumours. First, the cytotoxic effect of gene electrotransfer of both plasmids individually and combined was determined *in vitro* on B16F10 murine melanoma cell line. Two different pulse protocols were used; -ECT (electrochemotherapy;1300 V/cm, 100µs, 1Hz, 8pulses) and –GET (gene electrotransfer; 600 V/cm, 5ms, 1Hz, 8pulses). Cell viability was measured and the expression profile of IL-2 and IL-12 in cells was determined by real-time PCR and ELISA. Moreover, we also performed *in vivo* GET of pIL-2 and pIL-12 in B16F10 murine melanoma. Tumour growth delay was measured and the IL-2 and IL-12 concentrations in tumour and serum samples using ELISA were determined. In *in vitro* experiment, we demonstrated that the viability of cells treated with ECT pulses was significantly higher compared to the viability after treatment with GET pulses (p<0,05). In contrast, the expression of both interleukin genes in treated cells with ECT pulses was lower (p<0,05). After the administration of GET pulses *in vivo*, increased protein expression of IL-2 and IL-12 was observed in the respective groups and combination (pIL-12+pIL2) group (p<0,05). Tumour growth delay was observed in the GET pIL-12 and combination group and in some mice also complete tumour regression. Long-term anti-tumour immunity was observed in the combination group after tumour rechallenge.

To conclude, we demonstrated the anti-tumour effectiveness of gene therapy with IL-2 and IL-12 in murine B16F10 melanoma.

***Keywords**: chemokine, CCL5, CCL17, gene electrotransfer, irradiation, cytokine expression profile*

IZVLEČEK

Imunoterapija je postala pomemben pristop za zdravljenje raka. Primer je uporaba citokinov, kot sta interlevkin 2 (IL-2) in 12 (IL-12), ki neposredno stimulirata imunske celice na mestu tumorja. Ta citokina sta bila najprej omejena na uporabo v obliki rekombinantnih proteinov, danes pa ta pristop v večji meri nadomešča genska terapija. Cilj naše študije je bil določiti celično viabilnost, tumorsko rast in izražanje citokinov po genskem elektroprenosu plazmidov z zapisom za *Il2* (pIL-2) in *Il12* (pIL-12) v celicah in tumorju B16F10. Na celični liniji mišjega melanoma B16F10 je bil določen citotoksični učinek genskega elektroprenosa obeh plazmidov posebej in v kombinaciji. Uporabili smo dva različna protokola elektroporacije, in sicer-ECT (elektrokemoterapija; 1300 V/cm, 100 µs, 1Hz, 8pulzov) in -GET (genski elektroprenos; 600 V/cm, 5ms, 1Hz, 8pulzov). Odstotek viabilnosti je bil določen s pomočjo PrestoBlue™ reagenta, ekspresijski profil IL-2 in IL-12 pa z metodo PCR v realnem času in ELISA testom. Poleg tega je bil izveden tudi *in vivo* GET pIL-2 in pIL-12 na mišjem tumorskem modelu melanoma B16F10. V vzorcih tumorjev in seruma so bile s testom ELISA določene koncentracije obeh interlevkinov. V poskusu *in vitro* je bilo dokazano, da je viabilnost celic, tretiranih z ECT pulzi bistveno višja v primerjavi z viabilnostjo po tretiranju z GET pulzi ($p<0,05$). Nasprotno je bila ekspresija genov za interlevkina v celicah tretiranih z ECT pulzi nižja ($p<0,05$). V *in vivo* skupinah, kjer je bil izveden GET pIL-12 in pIL-2 samostojno ali v kombinaciji, je bila opažena povečana ekspresija IL-12 oz. IL-2 ($p<0,05$). Po terapiji z GET pulzi je bila pri skupinama pIL-12 in v kombinaciji opažena zakasnitev rasti tumorja v času, pri nekaterih miših pa tudi regresija tumorja. Po ponovnem injiciranju tumorskih celic je bila v skupini s kombinacijo opažena dolgoročna protitumorska imunost. V študiji smo tako dokazali protitumorsko učinkovitost genske terapije s pIL-2 in pIL-12 pri mišjem melanomu B16F10.

***Ključne besede:** elektroporacija, genska terapija, interlevkin 2, interlevkin 12, ekspresijski profil, ELISA*

A biomimetic *in vitro* infection model of porcine urothelium: Correlations between bacterial strains' pathogenicity level and cytokine production level with virulence-associated genes

Biomimetični *in vitro* infekcijski model prašičjega urotelija: Korelacije med ravnijo patogenosti bakterijskih sevov ter ravnijo produkcije citokinov in geni, povezanimi z virulenco

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ABSTRACT

Extraintestinal pathogenic *Escherichia coli* strains are capable of causing infections at various anatomical sites of the human body. The most frequent are urinary tract infections, which are predominantly caused by uropathogenic *E. coli* strains (UPEC). To understand the pathogenic processes of UPEC, many different models have been established so far, among them our biomimetic *in vitro* model of the porcine urothelium. The aim of our study was to use our biomimetic urothelial model to reveal correlations between the bacterial strains' pathogenicity level and cytokine production level with the presence of bacterial virulence-associated genes. A set of previously characterised bacterial *E. coli* strains, comprising human *E. coli* strains, isolated either from urine samples of patients with urinary tract infections or isolated from faecal samples of healthy individuals, was employed in infection assays performed on our biomimetic *in vitro* model. Based on the obtained results – drop in the viability of normal porcine urothelial cells (NPU), and fold change in total cytokine production by NPU cells compared to the negative control – bacterial strains' pathogenicity level and cytokine production level were determined. Correlations between pathogenicity level and cytokine production level with the presence of virulence-associated genes were analysed by an automated Fischer's exact test. Results revealed a positive correlation between the Highly pathogenic group of strains with the presence of following virulence-associated genes: *cnf1*, *hlyA*, *clbAQ*, *papGIII*, *sfaDE* and *tcpC*. Furthermore, a positive correlation was revealed between *E. coli* strains, causing a high fold change in total cytokine production with the presence of virulence-associated gene *cnf1*. Obtained results further strengthened the usability of our biomimetic urothelial infection model.

***Key words**: commensal E. coli, ExPEC, cell culture, viability, virulence*

IZVLEČEK

Zunajčrevesni patogeni sevi bakterije *Escherichia coli* lahko povzročijo okužbe različnih anatomskih mest človeškega telesa. Najpogostejše so okužbe sečil, ki jih pretežno povzročajo uropatogeni sevi *E. coli* (UPEC). Za razumevanje patogenih procesov sevov UPEC je bilo doslej vzpostavljenih veliko različnih modelov, med njimi tudi naš biomimetični *in vitro* model prašičjega urotelija. V naši raziskavi smo ugotavljali povezavo med ravnijo patogenosti bakterijskih sevov in ravnijo produkcije citokinov, ki sta bili določeni na biomimetičnem modelu *in vitro*, s prisotnostjo genov, povezanih z virulenco. V raziskavi smo uporabili nabor predhodno karakteriziranih sevov *E. coli*, ki je obsegal človeške seve *E. coli*, izolirane iz vzorcev urina bolnikov z okužbo sečil, in seve, izolirane iz vzorcev blata zdravih oseb. Na podlagi pridobljenih rezultatov – ugotovljenega upada viabilnosti normalnih prašičjih urotelijskih celic (NPU) biomimetičnega modela in spremembe v produkciji citokinov s strani celic NPU, v primerjavi z negativno kontrolo – smo določili ravni patogenosti sevov in ravni produkcije citokinov NPU celic. Korelacije med ravnijo patogenosti in ravnijo produkcije citokinov NPU celic s prisotnostjo bakterijskih genov, povezanih z virulenco, smo analizirani z avtomatiziranim Fischerjevim natančnim testom. Rezultati so pokazali pozitivno korelacijo visoko patogenih sevov s prisotnostjo naslednjih genov: *cnf1*, *hlyA*, *clbAQ*, *papGIII*, *sfaDE* in *tcpC*. Poleg tega je bila ugotovljena pozitivna povezava med sevi, ki so povzročili visoko raven produkcije citokinov s prisotnostjo gena *cnf1*. Pridobljeni rezultati so še dodatno potrdili uporabnost našega biomimetičnega *in vitro* infekcijskega modela.

***Ključne besede**: komenzalna E. coli, ExPEC, celična kultura, viabilnost, virulenca*

Implementation of an automatised approach to DNA sequencing data analysis

Implementacija avtomatiziranega pristopa k analizi podatkov DNA sekvenciranja

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ABSTRACT

In Next Generation Sequencing (NGS), genotype classification of individuals is based on calling variants. Our study included raw NGS analysis data from 24 individuals. The result is the VCF file (variant call format) containing the table of true positive potential variants in the variable filter. The PASS mark is often used for variants for which the neural network classifier gave a higher probability of a non-reference variant call than a reference, i.e. true positive variant call. We improved the protocol of DNA sequencing data analysis implementing MGPS v4.1 pipeline. Instead of the GATK Variant Caller v1.6. module in existing Illumina MiSeq tools, we used the Haplotype Caller module derived from the GATK v3.8, which is more accurate as it rejects the alignment data around the position of the suspected variant and re-reads data from that region. Nucleotide sequence alignment algorithm was upgraded from version 0.7.9 in existing protocols to 0.7.12. The upgrade includes pre-trimming of technical nucleotide sequences. Analysis of the number of called and PASS variants between the approaches was evaluated in the R software environment with the Wilcoxon signed-rank test. A statistically significant difference between the detected number of called variants and PASS variants of the upgraded and existing approach was observed. The upgraded approach detected on average 26-fold more called variants ($p = 3{,}061 \cdot 10^{-9}$) and 33-fold more PASS variants ($p = 6{,}202 \cdot 10^{-14}$). Out of 12 variants relevant for diagnosis, 5 positive PASS variants were missed by existing protocol (41.7 %), but not by our improved protocol.

***Key words**: NGS, bioinformatics, sequencing, Illumina MiSeq*

IZVLEČEK

Pri metodi sekvenciranja naslednje generacije (NGS) klasifikacija posameznikov za določen genotip temelji na določanju različic. Naša raziskava je zajemala analizo surovih NGS podatkov 24 posameznikov. Rezultat je VCF (ang. *variant call format*) datoteka, ki vsebuje tabelo potencialnih pravilno pozitivnih (ang. *true positive*, TP) različic oziroma kandidatnih genotipov. Oznaka PASS je pogosto uporabljena za različice za katere je klasifikator nevronske mreže podal višjo verjetnost nereferenčne določitve različice kot za referenco, tj. TP določitev različice. Z implementacijo MGPS v4.1 zaporedja ukazov

smo izboljšali pristop k analizi podatkov DNA sekvenciranja. V nadgrajenem protokolu smo namesto modula GATK Variant Caller iz različice v1.6. obstoječega orodja na aparatu Illumina MiSeq uporabili modul Haplotype Caller, pridobljen iz programskega paketa GATK v3.8., ki je natančnejši, saj zavrne podatke o poravnavi okoli mesta, kjer se sumi na različico in ponovno prebere odčitke v tej regiji. Prav tako smo nadgradili algoritem poravnave nukleotidnih zaporedij iz različice 0.7.9 v obstoječem protokolu na 0.7.12. Nadgradnja zajema tudi predhodno rezanje tehničnih nukleotidnih zaporedij. Analizo števila določenih različic in različic PASS med obema pristopoma smo ovrednotili v programskem okolju R z Wilcoxon-ovim statističnim testom predznačenih rangov, ki je pokazal statistično značilno razliko med odkritim številom določenih različic in različic PASS med nadgrajenim in obstoječim pristopom. Nadgrajen pristop je v povprečju odkril 26-krat več določenih različic ($p = 3{,}061 \cdot 10^{-9}$) in 33-krat več različic PASS ($p = 6{,}202 \cdot 10^{-14}$). Prav tako smo ugotovili, da je nadgrajen pristop odkril 5 pozitivnih različic PASS od 12 (41,7 %) pomembnih za diagnozo, ki jih je obstoječi zgrešil.

***Ključne besede**: NGS, bioinformatika, sekvenciranje, Illumina MiSeq*

Altered methylation patterns in *PIK3AP1* (BCAP) and *SPON2* (spondin-2) genes in patients with Periodic fever, aphthous stomatitis, pharyngitis and adenitis (PFAPA) syndrome

Spremenjena metilacija DNK v *PIK3AP1* (BCAP) in *SPON2* (spondin-2) genih pri pacientih s Periodičnim vročinskim sindromom z aftami, tonzilitisom in adenitisom

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ABSTRACT

Periodic fever, aphthous stomatitis, pharyngitis and adenitis (PFAPA) syndrome is the most common autoinflammatory disease in children and often grouped together with hereditary periodic fever syndromes, although its cause and hereditary nature remain unexplained. The rate of familial occurrence of PFAPA syndrome and the fact that similar syndromes are genetic both suggest that the cause could be hereditary, however, genetic research on PFAPA syndrome has not yet revealed causal changes. DNA methylation is also hinted at by the very property of the syndrome to disappear spontaneously by puberty. Since studies suggest that DNA methylation plays a role in other autoinflammatory diseases, we hypothesised that methylation patterns might be aberrant in at least a small portion of peripheral blood mononuclear cells in PFAPA patients. Epigenome analysis was performed using pooled DNA libraries enriched for methylated genomic regions using Methylated DNA Immunoprecipitation (MeDIP) and Methyl-CpG-binding domain (MBD). We identified candidate genes, of which two are known to be involved in the inflammation process, and further evaluated them using methylation-specific restriction enzymes coupled with qPCR (MSRE-qPCR). MSRE-qPCR proved to be a quick and reliable method for quantifying DNA methylation in selected regions. The analysis showed that changes in DNA methylation in the *PIK3AP1* and *SPON2* genes were present in patients with PFAPA syndrome. Both B cell adapter protein (BCAP, *PIK3AP1*), as a PI3K binding inhibitor of inflammation, and spondin-2 (*SPON2*), as a pattern recognition molecule and integrin ligand, are involved in autoinflammation and could influence the development of PFAPA syndrome.

***Key words**: PFAPA, differential methylation, PIK3AP1, SPON2, MSRE-qPCR, MeDIP, MBD*

IZVLEČEK

Periodični vročinski sindrom z aftami, tonzilitisom in adenitisom (sindrom PFAPA) spada v skupino avtoinflamatornih bolezni in je najpogostejši periodični vročinski sindrom v pediatrični populaciji. Večina avtoinflamatornih bolezni se deduje klasično, medtem ko PFAPA, ki si deli lastnosti s sorodnimi in genetsko opredeljenimi avtoinflamatornimi boleznimi, ostaja vzročno nepojasnjena bolezen. Pojavljanje sindroma PFAPA v nekaterih družinah in dejstvo, da so sindromu PFAPA podobni sindromi genetski, nakazuje, da bi lahko bil vzrok deden, vendar genetske raziskave pri sindromu PFAPA do sedaj še niso odkrile vzročnih sprememb. Na metilacijo DNA namiguje tudi sama lastnost sindroma, da do pubertete spontano izzveni, torej, da je mehanizem, ki bolezen vzdržuje prehodne narave. Študije metilacije DNA pri avtoinflamatornih procesih nakazujejo, da bi lahko le-ta vplivala na ekspresijo z vnetjem povezanih genov in na sam proces vnetja, zato smo pri bolnikih s sindromom PFAPA analizirali spremembe v metilaciji genomske DNA ter jih primerjali s kontrolno skupino zdravih otrok primerljive starosti. Uporabili smo dve metodi za obogatitev DNA za metilirane regije – MeDIP in MBD, rezultate pa potrdili z metodo MSRE-qPCR. MSRE-qPCR metoda se je izkazala kot hitra in zanesljiva metoda za kvantifikacijo metilacije DNA v izbranih regijah. Analiza je pokazala, da so pri pacientih s sindromom PFAPA prisotne spremembe v metilaciji DNA v genih *PIK3AP1* in *SPON2*. B celični adapterski protein (BCAP, *PIK3AP1*), kot PI3K vezavni inhibitor vnetja in spondin-2 (*SPON2*), kot vzorčno prepoznavna molekula in ligand integrina, sta vpletena v vnetne procese in bi lahko vplivala na razvoj sindroma PFAPA.

***Ključne besede**: sindrom PFAPA, metilacija DNK, PIK3AP1, SPON2, MSRE-qPCR, MeDIP, MBD*

Characterisation and identification of differentially expressed micro RNAs in hop plants (*Humulus lupulus* L.) infected with *Verticillium nonalfalfe*

Karakterizacija in identifikacija diferenčno izraženih miRNA v hmelju (*Humulus lupulus* L.) okuženim z *Verticillium nonalfalfae*

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ABSTRACT

MicroRNAs (miRNAs) are 20 to 24 nucleotides long non-coding RNAs that regulate gene expression at the post-transcriptional level. They are loaded into the RNA-induced silencing complex and lead it to a complementary sequence in the transcripts for cleavage. They are involved in the regulation of various biotic and abiotic stress responses of plants. To date, little is known about the miRNAs of hops and their role in response to *Verticillium nonalfalfae* infection. In our study, we characterised miRNAs in the hop cultivar, Wye Target, and identified differentially expressed (DE) miRNAs and their targets upon inoculation of hop plants with *V. nonalfalfae*. Hop plants were artificially inoculated with highly virulent *V. nonalfalfae* strain T2, and sampling of control and treated plants was performed 24 hours post-inoculation. In a miRNA-Seq analysis, we predicted 246 potential miRNA loci supported by the draft hop genome sequence. Of the predicted miRNAs, 57 miRNAs belong to 29 known miRNA families deposited in miRBase and 60 novel miRNAs derive from 110 different miRNA precursors. Using the NOISeq R package, we observed nine known and 33 novel miRNAs that show differential expression between control and treated hop plants upon inoculation with *V. nonalfalfae*. *In silico* target analysis predicted 147 transcripts to be targeted by DE miRNAs. The enriched functions of miRNA targets are associated with DNA binding, acetyl-CoA carboxylase activity and serine-type endopeptidase activity and are involved in various biological processes, e.g. auxin-activated signalling pathway, transsulfuration, protein retention in ER lumen and mRNA 3'-end processing.

***Key words**: Humulus lupulus, Verticillium nonalfalfae, fungal infection, biotic stress, microRNA*

IZVLEČEK

Mikro RNA (miRNA) so od 20 do 24 nukleotidov dolge, nekodirajoče RNA, ki uravnavajo izražanje genov na potranskripcijskem nivoju. Vežejo se v z RNA inducirani kompleks za utišanje genov in ga vodijo do komplementarnega zaporedja v transkriptih, ki jih nato razcepijo. miRNA so vključne v regulacijo različnih biotskih in abiotskih stresnih odzivov rastlin. V naši raziskavi smo karakterizirali miRNA v sorti

hmelja Wye Target in identificirali diferenčno izražene miRNA ter njihove tarčne transkripte po inokulaciji hmelja s fitopatogeno glivo *V. nonalfalfae*. Rastline hmelja smo umetno inokulirali z visoko virulentnim sevom *V. nonalfalfae* T2. Vzorčenje kontrolnih in okuženih rastlin smo izvedli 24 ur po inokulaciji. V analizi miRNA-Seq podatkov smo v genomu hmelja napovedali 246 potencialnih miRNA lokusov. Identificirali smo 57 miRNA, ki pripadajo devetindvajsetim znanim miRNA družinam iz podatkovne zbirke miRBase ter 60 novih miRNA, ki izhajajo iz 110 različnih miRNA prekurzorjev. Z uporabo paketa programskega jezika R, NOISeq, smo identificirali 9 znanih in 33 novih miRNA, ki kažejo diferenčno izražanje med kontrolnimi in z *V. nonalfalfae* okuženimi rastlinami. Za diferenčno izražane miRNA smo napovedali 147 tarčnih transkriptov. Obogatene funkcije miRNA tarč so povezane z vezavo na DNA, aktivnostjo acetil-CoA karboksilaze in aktivnostjo endopeptidaze serinskega tipa in so vključene v različne biološke procese, kot je na primer avksinska signalizacija, transsulfuracija, zadrževanje proteinov v lumnu ER in obdelava 3'-koncev mRNA.

***Ključne besede**: Humulus lupulus, Verticillium nonalfalfae, glivne okužbe, biotski stres, mikro RNA*

Regeneration of *Brassica napus* L. doubled haploids

Regeneracija podvojenih haploidov *Brassica napus* L.

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ABSTRACT

The developmental stage of the microspores at the time of culture initiation plays an important role in achieving success with microspore embryogenesis. The technique of isolated microspore culture is used to produce haploid and doubled haploid plants, which aim to create homozygous genotypes from heterozygous parental lines in only one generation. Since spontaneous chromosome doubling during microspore embryogenesis is rare in some plant species, such as *Brassica napus* L., chemicals are used to generate doubled haploids. A previous study showed that the most commonly used compound for chromosome doubling is colchicine, which inhibits the formation of spindle fibres during mitosis. Disturbance of normal polar chromosomal migration leads to chromosome doubling. In the present study, we tested different combinations of colchicine concentrations (0, 0.05, 0.5 mg/ml) and duration of treatments (12h, 18h, 24h) on the regeneration of plantlets and their ploidy level. After four weeks, the number of regenerated embryos in culture was counted. We discovered that the higher number of regenerated embryos was on the medium supplemented with 0.5 mg/ml colchicine, regardless of the duration. The ploidy level was analysed by flow cytometry and the percentage of regenerated haploid doubled haploid, tetraploid and mixoploid plants was assessed. The results showed that the higher number of regenerated doubled haploids were obtained by treating isolated microspores with 0.05 mg/ml colchicine for 24h. The regenerated plants were successfully acclimatised.

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

IZVLEČEK

Razvojna stopnja mikrospor v času iniciacije ima pomembno vlogo pri doseganju uspešne embriogeneze le teh. S tehniko izolacije mikrospor lahko regeneriramo haploidne in podvojene haploidne rastline, s katerimi je mogoče pridobiti popolnoma homozigotne linije iz heterozigotnih starševskih linij v samo eni generaciji. Ker je spontano podvojevanje kromosomov med mikrosporno embriogenezo pri nekaterih rastlinskih vrstah, kot je *Brassica napus* L., redko, se za pridobivanje podvojenih haploidov uporabljajo različne kemikalije. Študije so dokazale, da je kolhicin najpogosteje uporabljena kemikalija za podvojevanje kromosomov, saj 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 testirali vpliv različnih koncentracij kolhicina (0; 0,05; 0,5 mg/ml) v

kombinaciji z različnimi časi tretiranja (12h, 18h, 24h) na regeneracijo rastlin in njihovo ploidnost. Po štirih tednih se je preštelo število regeneriranih embrijev v kulturi. Ugotovili smo, da je bilo najvišje število regeneriranih embrijev na gojišču z dodanim 0,5 mg/ml kolhicina, ne glede na čas trajanja. Ploidnost se je izmerila s pretočno citometrijo, s pomočjo katere se je izračunalo delež regeneriranih haploidnih, podvojenih haploidnih, tetraploidnih in miksoploidnih rastlin. Rezultati so pokazali, da je bilo največ regeneriranih podvojenih haploidov iz tretiranj s koncentracijo 0,05 mg/ml kolhicina za 24h. Regenerirane rastline so bile uspešno aklimatizirane.

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

Virome status of Slovenian grapevine preclonal candidates as determined by HTS of Virus-Derived Small RNA

Analiza viroma slovenskih kandidatov klonske selekcije vinske trte s postopkom HTS virusnih malih RNA

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ABSTRACT

The presented research was focused on virus screening using high-throughput sequencing (HTS) technology, to get an overview of viruses and virus-like organisms that are present in preclonal candidates of Slovenian grapevine varieties. As a method of choice, small RNA sequencing was chosen. During the process of viral infection, the virus-derived small RNAs can be detected by deep sequencing of infected host plants. The isolation of small RNAs was performed by enrichment procedure using the "mirVana™ miRNA Isolation Kit" (Ambion, Life Technolgies), which enables that RNA molecules of <200 nt can be efficiently purified from the larger RNA species. Using the Ion Total RNA-Seq kit, miRNA libraries were constructed. Barcode-labelled miRNA libraries (cDNA libraries) were sequenced using the Proton ™ system (Ion Torrent ™; Life Technologies). After deep sequencing, open-source bioinformatics pipeline VirusDetect was employed, which can efficiently analyse small RNA datasets to identify both known and novel viruses. Together, 85 grapevine plants of 6 different cultivars were studied and six viruses and two viroids were identified: *Grapevine fanleaf virus* (GFLV), *Grapevine leafroll-associated virus 3* (GLRaV-3), *Grapevine rupestris stem pitting-associated virus* (GRSPaV), *Grapevine pinot gris virus* (GPGV), *Grapevine fleck virus* (GFkV), *Raspberry bushy dwarf virus* (RBDV), *Hop stunt viroid* (HSVd) and *Grapevine yellow speckle viroid 1* (GYSVd-1). The results of HTS analysis were confirmed by viruses specific RT-PCR and Sanger sequencing to allow an efficient validation of identified virus and viroid genome sequences.

Key words*: preclonal selection, grapevine viral diseases, high-throughput sequencing, VirusDetect*

IZVLEČEK

Raziskava je osredotočena na določanje virusov in virusom podobnih organizmov, ki so prisotni v kandidatih za klonsko selekcijo slovenskih sort vinske trte. Detekcija temelji na tehnologiji visokozmogljivega sekvenciranja (HTS; high-throughput sequencing) malih RNA. Med postopkom virusne okužbe je virusni genom v okuženih gostiteljskih rastlinah razrezan na male RNA,

ki jih je možno zaznati z visokozmogljivimi sekvenčnimi pristopi. Izolacijo malih RNA smo izvedli s postopkom obogatitve knjižnice z uporabo kita "mirVana ™ miRNA Isolation Kit" (Ambion, Life Technolgies), ki omogoča, da se molekule RNA <200 nt učinkovito ločijo od drugih razredov RNA. Z uporabo niza kemikalij "Ion Total RNA-Seq" so bile narejene knjižnice miRNA. Sekvenciranje smo izvedli na sekvenčnem sistemu Proton ™ (Ion Torrent ™; Life Technologies). Pridobljene sekvenčne podatke smo analizirali s pomočjo odprtokodnega programa VirusDetect, in tako *de novo* in/ali s pomočjo referenc sestavili kratka zaporedja RNA v daljša zaporedja, ki določajo posamezne viruse in viroide. Skupaj je bilo preučenih 85 rastlin vinske trte, ki pripadajo šestim različnim kultivarjem, identificirali pa smo 6 virusov in 2 viroida: *Grapevine fanleaf virus* (GFLV), *Grapevine leafroll-associated virus 3* (GLRaV-3), *Grapevine rupestris stem pitting-associated virus* (GRSPaV), *Grapevine pinot gris virus* (GPGV), *Grapevine fleck virus* (GFkV), *Raspberry bushy dwarf virus* (RBDV), *Hop stunt viroid* (HSVd) in *Grapevine yellow speckle viroid 1* (GYSVd-1). Rezultate analize HTS smo potrdili z RT-PCR, z uporabo virusno specifičnih začetnih oligonukleotidov in določanjem zaporedja s Sanger tehnologijo.

Ključne besede: *kandidati za klonsko selekcijo, virusne bolezni vinske trte, visoko zmogljivo sekvenciranje, VirusDetect*

Multimeric RNA species of viroids origin in hop plants

Vrste multimernih molekul RNA viroidov v hmelju

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ABSTRACT

Viroids of the *Pospiviroidae* replicate *via* an asymmetric rolling-circle mechanism with the help of enzymes provided by the host (Flores et al. 2009). The first step of this process is the iterative transcription of circular monomeric (+) RNA, which is catalysed by RNA polymerase II and produces oligomeric (–) RNAs that function as templates in the second step of the process for the synthesis of oligomeric (+) RNAs. After the third step, these are cut by RNase III into monomeric (+) RNAs, which are finally circularised by RNA ligase, resulting in mature viroids. Some indications of the nature of the replication intermediates come from research by Branch and Roberston (1984) and Hutchins et al. (1985), but some related questions still remain unanswered. In our work, we will use the state-of-the-art sequencing technology Nanopore for direct RNA sequencing of viroids, which we expect will give us a better insight into the exact length of the intermediates, the abundance of intermediates of different lengths, the relationship between viroid species and length of intermediates, etc. as well as new questions about how the viroids replicate.

***Key words**: viroids, rolling circle mechanism, oligomers, Nanopore RNA sequencing*

IZVLEČEK

Viroidi iz družine *Pospiviroidae* se podvajajo po asimetrični poti modela kotalečega se kroga, pri čemer vse korake v procesu katalizirajo gostiteljevi encimi (Flores et al. 2009). Prvi korak je iterativno prepisovanje krožne monomerne (+) molekule RNA, ki ga katalizira encim RNA polimeraza II. Pri tem nastajajo oligomeri (–) molekul RNA, ki v nadaljevanju delujejo kot matrica za sintezo oligomernov (+) molekul RNA. V tretjem koraku se ti oligomeri z encimom RNazo III pretvorijo v monomere (+) molekul RNA, nazadnje pa encim RNA ligaza zlepi skupaj konce monomerov, pri čemer se tvorijo končne krožne molekule (+) RNA, kar predstavlja zrel viroid. Kljub nekaterim dokazom o naravi intermediatov podvajanja, ki izhajajo iz raziskav Branch and Robertson (1984) ter Hutchins et al. (1985), ostaja nekaj vprašanj še vedno odprtih. V svojem delu bomo uporabili najsodobnejšo sekvenčno tehnologijo, Nanopore, za neposredno sekvenciranje molekul RNA viroidov. Predvidevamo, da nam bodo rezultati sekvenciranja, poleg odpiranja novih vprašanj o naravi podvajanja viroidov, tudi izboljšali vpogled v dolžino intermediatov, zastopanost intermediatov različnih dolžin,

razmerje med vrsto viroida in dolžino intermediatov.

Ključne besede: *viroidi, mehanizem kotalečega se kroga, oligomeri, intermediati, Nanopore sekvenciranje RNA*

Different post-genotyping procedures of dataset formation affect the allelic diversity measures in goat breeds

Različni postopki oblikovanja setov po genotipizaciji vplivajo na meritve alelne raznolikosti pri pasmah koz

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ABSTRACT

Allelic-diversity measures are good predictors for a long-term adaptation potential of the breeds. Information regarding the evolutionary response to environmental changes is therefore very important, especially for critically endangered local breeds and in the face of rapid climate and social changes. When assessing the allelic diversity estimates, the first step is to prepare animal datasets. A commonly used approach is the removal of highly related animals (one-step approach). Here we propose an alternative procedure, which firstly removes admixed animals showing stronger genetic relationships with other breeds and than in the second step, removes highly related animals in the breed (two-step approach). Our aim was to investigate how different dataset preparation procedures affect the allelic diversity parameters of 23 Alpine goat breeds, including Slovenian local and endangered Drežnica goat. All breeds were genotyped with Illumina Goat SNP50 BeadChip. Datasets constructed by one- or two-step approach were further used for calculating the mean allelic richness (mAR), a parameter that accounts for differences in the sample size. We detected and removed the admixed animals in 21 breeds. In comparison with the one-step approach, analyses of datasets using two-step procedure resulted in decreased mAR (0.2-11.3%) in the majority of breeds (14). In these breeds, mAR can be overestimated when analysed by one-step approach, due to the presence of additional foreign alleles from admixed animals. After removing these admixed animals, we obtained mAR values from more representative animals, which are lower in most cases. We suggest that removing admixed animals is a necessary additional step to obtain objective allelic diversity estimates to help guide breeding and conservation programs.

***Key words**: allelic diversity, adaptation, local goat breeds, Drežnica goat, SNP array*

IZVLEČEK

Stopnja alelne raznolikosti je dober pokazatelj prilagoditvene sposobnosti pasem na dolgi rok. Podatki o evolucijskem odzivu pasem na spremembe okolja so zato zelo pomembni za kritično ogrožene avtohtone pasme v obdobju hitrih podnebnih ter družbenih sprememb s katerimi se soočamo. Ključen korak pri

ocenjevanju alelne raznolikosti je priprava setov živali. Najpogosteje uporabljen pristop je odstranjevanje zelo sorodnih živali (enostopenjski pristop). Kot alternativa temu predlagamo postopek, kjer najprej odstranimo živali, ki kažejo močnejše genetske povezave z drugimi pasmami in nato v drugem koraku odstranimo zelo sorodne živali v pasmi (dvostopenjski pristop). Naš cilj je bil preveriti, ali različni postopki priprave setov živali vplivajo na parametre alelne raznolikosti 23 alpskih pasem koz vključno s slovensko ogroženo avtohtono drežniško kozo. Pasme so bile genotipizirane z Illumina Goat SNP50 BeadChip. Z eno- in dvostopenjskim pristopom smo oblikovali dva ločena seta živali in ju uporabili za izračun povprečnega bogastva alelov (mAR) s postopkom, ki upošteva razlike v velikosti vzorcev. Živali, ki imajo primesi drugih pasem, smo našli in odstranili pri 21 pasmah. V primerjavi z enostopenjskim pristopom, se je pri večini pasem (14) zmanjšalo mAR (0,2-11,3%) na podlagi dvostopenjskega pristopa. Z enostopenjskim pristopom lahko precenimo mAR pasem zaradi prisotnosti dodatnih tujih alelov od živali z večjim deležem primesi drugih pasem. Po njihovi odstranitvi smo dobili vrednosti mAR, ki so v večini primerov nižje, vendar so ocenjene na reprezentativnih živalih za določen pasmo. Glede na rezultate predlagamo odstranjevanje živali, ki imajo primesi drugih pasem, kot nujen dodaten korak za pridobitev objektivnih ocen alelne raznolikosti, ki predstavljajo smernice za oblikovanje rejskih programov.

***Ključne besede**: alelna raznolikost, prilagoditev, avtohtone pasme koz, drežniška koza, SNP mikromreža*

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