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TWINNING ON DNA-BASED CANCER VACCINES
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Symposium

TARGETS OF IMMUNOTHERAPY OF CHRONIC VIRAL INFECTIONS AND CANCER

Rīga Stradiņš University, Dzirciema iela 16, Rīga, Latvia

Senate Hall, Block K

May 24-26, 2016

ABSTRACT BOOK

Day 1, May 24, 2016

08.30 – 9.10 REGISTRATION

9.10 - 09.20 WELCOME AND OPENING

Uldis Berkis, the Director of the Research Department of Riga Stradins University

SESSION I: TARGETS OF IMMUNOTHERAPY OF CHRONIC VIRAL INFECTIONS

Chairs: Uldis Berkis, Elena Kashuba

09.20 - 10.00 Ingemar Ernberg (Karolinska Institutet, Stockholm, Sweden) What is the most efficient immunotherapy to Epstein-Barr virus (EBV) infection?

10.00 - 10.40 Irina Sominskaya (Biomedical Research and Study Center, Riga, Latvia) HBV - viral vaccine and the first vaccine against cancer.

SESSION I: TARGETS OF IMMUNOTHERAPY OF CHRONIC VIRAL INFECTIONS (continued)

Chairs: Uldis Berkis, Elena Kashuba

11.00 -11.30 Maria Isaguliants (Riga Stradins University, Riga, Latvia, Gamaleya Research Center of Epidemiology and Microbiology, Moscow, Russia, and Karolinska Institutet, Stockholm, Sweden) Naked DNA as a vehicle in immune prevention and immune therapy of viral infections.

11.30 - 11.45 Andris Dishlers (Biomedical Research and Study Center, Riga, Latvia) A novel efficient HBV vaccine candidate based on HBc and preS1 components presented by VLPs.

11.45 - 12.00 Juris Jansons (Biomedical Research and Study Center, Riga, Latvia) Immunogenicity in mice of plasmid DNA encoding HCV core and alternative reading frame proteins.

SESSION I: TARGETS OF IMMUNOTHERAPY OF CHRONIC VIRAL INFECTIONS (continued)

Chairs: Ingemar Ernberg, Maria Isaguliants

13.20 - 14.00 Britta Wahren (Karolinska Institutet, Stockholm, Sweden) Nanovaccine HIV: prophylactic and therapeutic DNAs.

14.00 - 14.40 Lars Frelin (Karolinska Institutet, Stockholm, Sweden) Targets for HCV prophylactic and therapeutic vaccines.

14.40 - 15.00 Stefan Petkov (Karolinska Institutet, Stockholm, Sweden) Delivery of naked DNA for immunization and gene therapy: optimization using human explant model and reporter genes.

WHAT IS THE MOST EFFICIENT IMMUNOTHERAPY TO EPSTEIN-BARR VIRUS (EBV) INFECTION?

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Background

Epstein-Barr virus (EBV) is the most common pathogenic virus in the human population with a prevalence globally >90 %. As a herpesvirus it remains latent after primary infection throughout life. Every year EBV is involved in the pathogenesis of some 200.000 new cancers, affecting the immune system, nasopharynx or stomach. The viral carcinogenesis depends on cofactors such as genetic, co-infection or immunologic (e.g. immunosuppression). In healthy people primary infection often results in infectious mononucleosis (IM), a self-limiting lymphoproliferative disease, particularly if it is delayed to adolescence.

Aims

We study EBV-related complications and cancers in HIV-infected patients, transplant patients and in the Chinese population with high risk of nasopharyngeal cancer (NPC; 1-5). In this lecture the most appropriate type of immunotherapy against EBV-infection and its complications will be discussed.

Results

Restoration of host cellular immunity and/or adoptive T-cell therapy has been successfully employed to block post-transplant lymphoproliferative disease (PTLD) and lymphomas in immunosuppressed patients (3-5). NPC is usually treated with radio- and chemotherapy. In the future NPC should be subject to immunotherapy trying checkpoint inhibitors.

Discussion

There is an efficient vaccine to EBV directed against its main receptor ligand gp 350. However it is hard to define a target population for cost-effective preventive vaccination, given the wide dissemination of the virus, the nature of primary infection and of the host-virus interaction. On the other hand immune based therapies will mot likely gain increased attention.

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HBV - VIRAL VACCINE AS THE FIRST VACCINE AGAINST CANCER

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Persons who become infected with HBV either recover from their infection in several months or they may remain chronically infected for most of their life. Persons with chronic HBV infection are at high risk of death from cirrhosis and liver cancer. In addition, they are likely to transmit their infection to other people. Because hepatitis B causes liver cancer, it is considered the first cancer vaccine.

Hepatitis B vaccine provides protection against infection with HBV by producing immunity or antibodies to the surface protein or outer coat of the virus. This outer coat is called hepatitis B surface antigen or HBsAg. The first HBV vaccines to be used in humans involved injection of empty 22-nm subviral particles purified from the plasma of chronic carriers. This vaccine was produced by Merck and at the same time by the Pasteur institute. Subsequently, it was approved by the FDA in 1981. The second generation of HBV vaccines consisted of similar subviral particles, which are produced by recombinant DNA technology as recombinant proteins in stably transfected eukaryotic cell lines (Engerix B and Recombivax HB). These vaccines have gradually replaced the first-generation plasma-derived vaccines and are currently used for universal vaccination of newborns and adults in >170 countries worldwide Third-generation HBV vaccines containing one (Pre-S2) or two (Pre-S1 and Pre-S2) additional HBV envelope proteins have been developed in Germany, France, Korea and Israel in transfected mammalian cells.

Hepatitis B vaccine provides greater than 90 percent protection to infants, children, and adults immunized before being exposed to the virus. The efficacy of plasma-derived and recombinant hepatitis B vaccine in preventing acute and chronic infection has been demonstrated in controlled clinical trials conducted with adults, children, and infants. In addition, a number of studies have examined various vaccination schedules and dosages and all have documented short-term vaccine safety. What can be done to improve protection by the current yeast-derived vaccine? It is possible to change dose, use more aggressive adjuvants, novel delivery methods (vectors, VLPs etc.), and improve antigen composition.

NAKED DNA AS A VEHICLE IN IMMUNE PREVENTION AND IMMUNE THERAPY OF VIRAL INFECTIONS

Maria Isaguliants [1,2,3*], Elizaveta Starodubova [2,4,5], Stefan Petkov [2], Athina Kilpeläinen [2], Olga Krotova [2,3,4], Juris Jansons[1,6], Oleg Latyshev [3,5], Amir Tukhvatullin[3], Denis Logunov [3,5]

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DNA-based vaccines are widely used in prophylactic and therapeutic attempts to treat numerous infectious diseases, malignancies, allergies and autoimmunity. They are stable, easy to construct and manipulate via custom gene synthesis, straightforward to produce at high purity, and most importantly safe, since they produce non-multiplying (non-live, non-replicating) antigens [1]. The main advantage of DNA vaccines is expression of the correctly processed and folded immunogens in/by the host cells which provides for their efficient delivery to the antigen presenting cells, and ensures the induction of both T-cell and B-cell responses. Anti-viral DNA vaccines employ a selection of viral genes which, when expressed in the host cells, are expected to induce an immune response preventing viral infection in prophylactic, or exterminating already infected cells in the immunotherapeutic applications. Biological functions carried by viral proteins, often adverse, (immuno)modulating or directly hazardous, have to be taken into account in anti-viral vaccine design. Multiple approaches exist for viral gene optimization including truncation, deletion of harmful domains & signatures, domain shuffling, insertion of exogenous signals of antigen processing. These technqiues are applied in addition to the standard approaches of optimization of codon usage and mRNA folding. Numerous preclinical and clinical trials have demonstrated that DNA vaccines are well tolerated, do not trigger major adverse effects and can induce an immune response of desired specificity. However, the potency of these responses in humans, as well as in larger animals and primates appears to be much lower than in small laboratory animals [2,3]. One of the strategies to overcome low immunogenicity of this vaccine modality as an enhancement of plasmid delivery by *in vivo* electroporation (EP) which can enhance gene uptake and expression by up to 1000-fold compared to un-aided DNA injections[1]. Application of EP augmented cellular and humoral immune responses in small, as well as large animal models. Series of clinical trials performed lately using improved methods of DNA delivery demonstrated the potential of the genetic immunotherapy to treat chronic viral infections with hepatitis C, hepatitis B, human papilloma viruses. Our research group develop this methodology as a complement to antiretroviral therapy in HIV-1 infection in order to prevent or hinder the development of drug resistance. Promising results in recent applications of DNA vaccines highlight their perspectives as immunoprophylactic as well as immunotherapeutic remedies against acute and chronic viral infections.

Acknowledgements: Supported by BALTINFECT. Experimental studies in DNA vaccination against drug resistance in HIV infections are supported by the Russian Science Foundation grant 15_15_30039. Mobility of researchers is supported by the Thematic partnership grant of the Swedish Institute 09272_2013 and training, by the Horizon 2020 project VACTRAIN 692293.

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A NOVEL EFFICIENT HBV VACCINE CANDIDATE BASED ON HBC AND preS1 COMPONENTS PRESENTED BY VLPs

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Background: Despite the availability of an effective prophylactic vaccine, more than 350 million humans are chronically infected with HBV worldwide being at risk to develop liver cirrhosis or hepatocellular carcinoma. Chronic hepatitis B causes 80% of all liver cancer, which is the 9th leading cause of death. Therefore, a vaccine that protects against hepatitis B infection also helps to prevent the liver cancer. However, application of existing HBV vaccines is limited to prophylactic vaccination and not for use in chronically infected HBV patients. To induce T-cell response CTL-epitope rich HBc antigen of HBV can be regarded as a putative component of therapeutic HBV vaccine. The chimeric HBc-preS1 VLPs may serve as prototypes for the generation of a combined therapeutic and prophylactic anti-HBV vaccine. In *E. coli*-cells HBc shows high-level expression and correct self-assembly in VLPs. It has been shown previously that anti-preS1 antibodies are virus-neutralising and thus HBc-preS1 based vaccine could be used as a new type vaccine with universal application.

Aims: The present work is targeted to the development of a novel efficient HBV vaccine based on HBc and preS1 as essential vaccine components which are put together in one module and each of them being responsible for therapeutic and profilactic vaccine response, respectively.

Results: After insertion of a functional preS1 fragment in selected sites of HBc, HBc-preS1 VLPs were expressed in *E-coli* on high degree, purified and subjected to structural and immunological studies. The preS1 stretch showed high antigenicity and superficial localization on VLPs. After immunization of BALB/c mice, specific T-cell activation and a high-titer antibody response against the pre-S1 epitope were found.

Acknowledgements: We thank Mrs. Inara Akopjana for performing the *E. coli* transformations and preparing the bacteria for HBc VLP purification and Mrs. Irina Stahovska for excellent technical assistance. Study was carried with the financial support by a European Regional Development Foundation Grant 2013/0053/2DP/2.1.1.1.0/13/APIA/VIAA/006.

IMMUNOGENICITY IN MICE OF PLASMID DNA ENCODING HCV CORE AND ALTERNATIVE READING FRAME PROTEINS

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Background: The nucleocapsid (core) protein of HCV represents an attractive target for an HCV vaccine, but the efforts to make it immunogenic had only limited success. We attempted to delineate the factors restricting the immunogenicity of HCV core, and improve its performance as DNA immunogen in mice. Besides the core protein, the 5' terminal part of HCV genome encodes a frameshift core+1/ARFP protein, the role of which in HCV life cycle is yet unclear. In the previous study we analyzed the immunogenicity in rabbits of core aa 1-173, 1-152, 147-191, and of its main alternative reading frame product ARFP (*Sominskaya I et al, 2015*). A strongest antibody response was obtained against both HCV core and ARFP (titers of 10^6), indicating that they may compete in the induction of immune response in DNA-immunization made with viral genes allowing the frameshift.

Aims: To characterize the immunogenicity of plasmids expressing proteins encoded by the 5´terminus of HCV RNA in DNA-immunization, and define the correlates of immunogenicity.

Materials and Methods: Plasmids carrying cDNA encompassing the 5´terminus of HCV 1b genome encoding HCV core and ARFP, were obtained by cloning. Plasmids were transfected into eukaryotic cells, and expression level of HCV core and ARFP was evaluated by Western blotting with anti-core and anti-ARFP rabbit antibodies. Groups of BALB/c mice (n=5) were immunized twide with 40 ug plasmids or empty vector intradermally with four days interval; injections were followed by electroporation (BEX, Japan). After 21 days, mice were sacrificed, spleens and sera were collected. Cellular responses in spenocytes were analyzed IFN-γ/IL2 Fluorospot after stimulation with HCV core and ARFP and antigen-derived peptides. Antibody response was assessed by ELISA.

Results: To elucidate the role of ARFP in induction of immune response against core protein, a set of plasmids carrying variants of the 5' terminus of HCV genome were constructed: (1) plasmid carrying wild type sequence encoding core aa 1-191 protein with allowed co-expression of ARFP; (2) plasmid expressing core aa 1-191 protein with prohibited expression of ARFP; and (3) plasmid with prohibited expression of HCV core but expressing ARFP via the frame-shift mechanism. It was shown that prohibition of expression of ARFP by single nucleotide mutations significantly increases the expression of HCV core protein. The expression of ARFP protein remains low all cases. The cellular immune responses against the epitopes of HCV core proteins were similar for the plasmid co-expressing core and ARFP, and the plasmid with prohibited expression of ARFP. A weak antibody response against HCV core epitopes was registered in both cases. None of the immunizations induced either cellular, or antibody response against ARFP.

Discussion: Immunization with HCV core genes induced low specific immune response. Both natural and mutated HCV core genes with prohibited frame-shift provide the same levels of specific cellular and antibody responses. Thus, a higher expression of HCV core from the mutated gene compared to the wild type sequence could not provide for its better immunogenicity. Efficacy of ARFP expression by the natural ribosome frameshift mechanism was low and obviously insufficient to induce a specific immune response in DNA-immunization. Thus, anti-ARFP immune response is not competing with that against HCV core, and cannot explain low immunogenicity of the latter in DNA-immunization performed with the virus-derived genes.

Acknowledgements: Authors acknowledge financial support from the projects BALTINFECT, VACTRAIN, Research Council of Latvia 532/2015, and Swedish Institute 09272_2013.

NANOVACCINE HIV, PROPHYLACTIC AND THERAPEUTIC DNAs

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HIV plasmid nanovaccines are under clinical development for prophylaxis and treatment of HIV/AIDS. They contain plasmid DNA (pDNA) consisting of only CpG motifs asdjuvant and are developed to be delivered by electroporation or by needle-free devices to skin cells. To develop a stable, commercializable nanovaccine that maintains the structure and physico-chemical properties of the desired HIV plasmid DNAs, we have developed several plasmid mixtures, encoding either the env, gag or RT specificities of HIV-1, following a finding of immunocompetence with a full mix of plasmids. However, delivery of these nanoplasmids has permitted overriding both dose limitation as well as immunodominance limitation by delivery of the plasmids by needlefree devices, Bioject and/or an electroporation device. It is generally accepted that the efficiency of plasmid DNA-based drugs is related to a higher level of structural forms of pDNA. The stability of the plasmid DNA and the extent of its biological activity is the amount of supercoil forms compared to the open circular forms, which are generated due to a chemical or physical degradation force. We therefore investigated the effect of the chemical environment and shearing forces on the stability of biologically active superhelical forms of pDNA. In addition we have been able to study in vivo continuous expression of immune nanoplasmid DNA by IVIS, and the resulting immune reactivity by the plasmid-expressed genes. These concerns resulted in an optimal delivery schedule for gene immunization, in this case anti-HIV immune responses.

TARGETS FOR HCV PROPHYLACTIC AND THERAPEUTIC VACCINES

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Background: Chronic hepatitis C virus (HCV) infection is a major causative agent for severe liver disease and cancer worldwide. Globally, around 130-170 millions are chronic carriers of the virus. Importantly, the introduction of direct-acting antivirals (DAA) has revolutionized the treatment with cure rates above 90 % in most patient groups. Thus, the urgent need for a HCV vaccine has markedly reduced. However, the DAA treatment is associated with high costs, it does not fit all patient groups, problem with resistance development, side effects, and the DAAs does not protect against re-infection. Thus, even though highly efficient treatments are available, there is an unmet need to prevent the spread of HCV through a vaccine. In addition, a therapeutic vaccine will support eradication of HCV infection and to broaden the HCV post-cure T cell response to reduce the risk of re-infection.

Aim: To define potential targets for HCV prophylactic and therapeutic vaccines.

Results: Currently there are no prophylactic or therapeutic vaccines available for HCV. However, numerous vaccines have been developed and tested for efficacy in pre-clinical and clinical trials. Both conventional and experimental vaccines have been tested such as recombinant proteins, peptides, virosome-peptide formulations, DNA, DNA-recombinant proteins, adenovirus vectors, MVA vectors, iscomatrix, yeast proteins, prime-boost approaches using DNA-protein, DNA-MVA[1], MVA-Adeno, and Adeno-Adeno. In addition, several different delivery techniques have been developed and utilized such as the gene gun, micro needles, biojector, *in vivo* intracellular injection (IVIN)[2] device, and *in vivo* electroporation[3]. In general, the clinically evaluated HCV vaccines have shown limited effects on HCV viral load and inefficient activation of HCV-specific immune responses. Importantly, from each study we have gathered new knowledge, which can be used in the design of future trials. Hence, the current available pre-clinical and clinical data can guide us in how to design a highly efficient HCV vaccine.
Toady, a few HCV vaccines are currently being tested for both protective and therapeutic effects.

Discussion: To successfully develop a prophylactic and/or therapeutic vaccine for HCV the following aspects have to be taken into consideration: 1) wisely choose a highly immunogenic vaccine antigen, 2) use of an efficient delivery technique, 3) inclusion of potent molecular adjuvants, and 4) a carefully designed clinical trial. All these aspects will be discussed.

Acknowledgements: This work was supported by grants from the Swedish Research Council, the Swedish Society of Medicine, the Åke Wiberg Foundation, and from Karolinska Institutet.

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OPTIMIZATION OF NAKED DNA DELIVERY IN HUMAN EXPLANT MODEL USING REPORTER GENES

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Background: Optical imaging of deep tissues in mammals is greatly facilitated by the recently developed family of near infra-red (NIR) fluorescent proteins (FPs) that absorb and fluoresce in a tissue transparency "optical window" between 650-900 nm. Earlier, we have used bioluminescent reporters to follow up the delivery and immunogenic performance of naked DNA vaccines[1]. iRFP670[2] was engineered from the PAS-GAF domains of the *Rp*BphP2 bacterial phytochrome of the photosynthetic bacterium *Rhodopseudomonas palustris* by introducing 14 amino acid mutations[2]

Aims: To investigate the applicability of NIR FPs as reporters for gene delivery.

Results Having shown that intradermal (ID) delivery of DNA immunogens is superior to intramuscular in induction of both cellular and antibody responses[1], we inoculated BALB/c mice with iRFP670 DNA by ID injection with subsequent electroporation (EP). EP was performed using *in vivo* electroporator CUY21EDITII (BEX Ltd, Japan) with multi-needle, two-needle, plate or plate-fork electrodes. iRFP670 expression was confirmed both *ex vivo* and *in vivo* using NIR optical imaging (Spectrum CT). *In silico* study of the proteasomal cleavage and MHC I binding properties of iRFP670 predicted a set of T cell epitopes forming four major clusters[3]. Representative peptides were synthesized and together with recombinant iRFP670 used for screening of immune response raised in iRFP670 gene recipients. A dominant epitope was localized at amino acids 224-257. Respective peptide induced the production of IFN-γ (100-300 SFC/10^6 splenocytes) in >50% of immunized mice. An anti-iRFP670 response was mainly cellular: the average titer of anti-iRFP670 antibodies did not exceed a titer of 10^3. The immunogenicity of iRFP670 was similar to that of firefly luciferase, and likewise not sufficient to clear the iRFP670-expressing cells from the sites of gene injection. The fluorescence from the iRFP670 gene injection sites was detectable 1 h post injection and persisted for 4 weeks (day 27 post injection) while decreasing by that time by 30% only. During the same period of time, the expression of the firefly luciferase usually decreases by up to 90%, and of the luciferase gene delivered with a potent gene immunogen by nearly 99%.

Discussion: The properties of iRFP670 properties, such as the NIR emission/excitation spectrum, low immunogenicity and substrate independence, make it an attractive alternative to bioluminescent reporters, and suitable for a longitudinal follow-up of the labelled cells in a variety of applications including gene immunization.

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Day 1, May 24, 2016

SESSION II: INVASIVE PROPERTIES AND PROGNOSIS OF CANCER, AND p53

Chairs: Ilze Strumfa, Galina Selivanova

15.20 - 15.30 Inese Drike (Riga Stradins University, Riga, Latvia) Modified Klintrup-Makinen inflammation score in relation to invasive properties of colorectal cancer.

15.30 - 15.40 Lubov Kolomencikova (Riga Stradins University, Riga, Latvia) Parafibromin: a reliable molecular marker in parathyroid neoplasms.

15.40 - 15.50 Inese Drike (Riga Stradins University, Riga, Latvia) p53 protein expression and its correlation with cell proliferation in colorectal cancer.

15.50 - 16.10 Arvids Jakovlevs (Riga Stradins Universitet, Riga, Latvia) 'p53 protein as a potential target of cancer vaccines in glioblastomas.

16.10 - 16.20 Mareks Marcuks (Riga Stradins Universitet, Riga, Latvia) Expression of aberrant p53 protein in gastric cancer

16.20 - 16.40 Poster presentations (5 min, 3-5 slides)

Lubov Kolomencikova, Kirsakmens G, Franckevica I, Abolins A, Prieditis P, Strumfa I. p53 protein expression is a rare event in parathyroid neoplasms.

Dzeina Mezale, Strumfa I, Vanags A. Expression of aberrant p53 protein in hepatocellular carcinoma.

Agita Jukna, Strumfa I, Vanags A, Gardovskis J. Expression of p53 protein in lung cancer.

Gatis Kirsakmens, Franckevica I, Kolomencikova L, Jakovlevs A, Balodis D, Strumfa I. Expression of aberrant p53 protein in medulloblastoma.

MODIFIED KLINTRUP-MAKINEN INFLAMMATION SCORE IN RELATION TO INVASIVE PROPERTIES OF COLORECTAL CANCER

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Background The progression of colorectal cancer and patient's survival depends on many factors, including the characteristics of peri- and intra-tumoural inflammation [Klintrup *et al*., 2005; Roxburgh *et al*., 2009]. Inflammatory infiltration into invasive margin of tumour has been associated with increased survival [Richards *et al*., 2012].

Aims The aim of our study was to evaluate inflammation in colorectal cancer in relation to the manifestations of invasive tumour growth.

Results The study included 262 consecutive retrospective cases of colorectal cancer, subjected to microscopic evaluation and statistical analysis. There were 132 (50.4% [95% confidence interval (CI): 44.4 – 56.4]) pT3 tumours, 85 (32.4% [27.1 – 38.3]) pT4 cancers and 40 (15.3% [11.4 – 20.1]) pT2 cases. After qualitative assessment of Klintrup-Makinen overall peritumoural inflammation score, the identified groups (no inflammation *versus* mild *versus* moderate *versus* severe inflammation) were distributed into two classes: low-grade (no or mild inflammation) *versus* high-grade (moderate or severe) inflammation. Low-grade inflammation was seen in 62 (46.9% [38.7 – 55.4]) pT3 and 51 (60.0% [49.4 – 69.8]) pT4 cancers while high-grade inflammation was found in 25 (62.5% [47.0 – 75.8]) pT2; 70 (53.0% [44.5 – 61.3]) pT3 and 34 (40.0% [30.2 – 50.6]) pT4 tumours. In cancers characterised by low-grade inflammation, lymphatic invasion was identified in 66.1% [57.5 – 73.8], intraneural invasion in 29.9% [22.6 – 38.4], and perineural invasion in 55.9% [47.2 – 64.2] of cases. In tumours showing high-grade inflammation, lymphatic invasion was seen in 51.8% [43.5 – 60.1], intraneural invasion in 22.9% [16.7 – 30.7], and perineural invasion in 40.7% [32.8 – 49.2] of cases. Chi-square test revealed statistically significant (defined as $p < 0.05$) difference between tumours exhibiting low-grade *versus* high-grade inflammation regarding lymphatic ($p = 0.019$) and perineural invasion ($p = 0.014$), but there was no statistically significant difference in case of intraneural ($p = 0.201$) invasion.

Discussion In colorectal cancer, high-grade peritumoural inflammation is statistically significantly related to less frequent manifestations of invasive growth including lymphatic and perineural invasion. Thus, assessment of these parameters indirectly indicates that inflammation could be a beneficial prognostic factor. Hypothetically, enhancement of inflammatory reaction by anti-cancer vaccination also could be favourable. The trend to less frequent occurrence of pT4 tumours in the class of colorectal cancer showing high-grade inflammation tends to reject hypothetic link between the assessed peritumoural inflammation and more advanced local tumour spread causing bowel obstruction.

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PARAFIBROMIN: A RELIABLE MOLECULAR MARKER IN PARATHYROID NEOPLASMS

Kolomencikova L, Franckevica I, Jukna A, Drike I, Bogdanova T, Abolins A, Strumfa I

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Background Nowadays, increasing number of patients is diagnosed with primary hyperparathyroidism due to the increased availability of routine automated serum calcium measurements, parathyroid hormone testing and screening for osteoporosis (DeLellis, 2011). Surgery is increasingly used for the treatment of primary hyperparathyroidism as it is considered safe and effective. Thus, there is increasing demand for exact morphological differential diagnostics between parathyroid carcinoma and benign mass lesions that can be complicated. Molecular markers, including parafibromin, are promising but still controversial (Juhlin *et al*., 2006; DeLellis, 2011; Gill, 2014).

Aims The aim of this study was to assess the expression of parafibromin in parathyroid carcinoma in comparison to adenoma and primary parathyroid hyperplasia.

Results A retrospective analysis of 134 surgically removed parathyroid glands was performed including 102 adenomas; 27 cases of primary parathyroid hyperplasia and 5 carcinomas. The expression of parafibromin was assessed by immunohistochemistry and subsequently evaluated as a qualitative binary estimate: complete loss of nuclear expression *versus* presence of nuclear reactivity. The rate of parafibromin expression was 0/5 in carcinoma (0.0%; 95% confidence interval (CI): 0.0 – 48.9); 102/ 102 in adenoma (100.0%; 95% CI: 95.6 – 100.0) and 26/ 27 in primary parathyroid hyperplasia (96.3%; 95% CI: 80.2 – 100.0). Thus, loss of nuclear parafibromin expression has sensitivity 100.0% (95% CI: 47.8 – 100.0), specifity 99.2 (95% CI: 95.8 – 100.0), positive predictive value 83.3% (95% CI: 35.9 – 99.6) and negative predictive value 100.0% (95% CI: 97.2 – 100.0).

Discussion In parathyroid disease, loss of nuclear parafibromin expression is a reliable diagnostic marker to distinguish between parathyroid carcinoma and benign mass lesions. The reported controversies can be at least partially attributable to technological differences and nuclear, nucleolar or cytoplasmic location of reactivity (DeLellis, 2011; Juhlin *et al*., 2006; Gill, 2014).

Acknowledgement The research was carried out within the frames of scientific project 08/2013, supported by Riga Stradins University.

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p53 PROTEIN EXPRESSION AND ITS CORRELATION WITH CELL PROLIFERATION IN COLORECTAL CANCER

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Background Colorectal cancer is among the most frequent malignancies globally and in the Western world. Cell proliferation is one of the indicators that show tumour progression rate and can be associated with worse prognosis of colorectal cancer [Valera *et al*., 2005]. p53 protein as a cell cycle regulating factor also has an important role in pathogenesis of colorectal cancer, leading to abnormal cell division [Goergescu *et al*., 2007]. There are still doubts whether there is correlation between Ki-67 and p53 expression in malignancies [Lumachi *et al*., 2012]

Aims The aim of our study was to evaluate p53 and Ki-67 protein expression in colorectal cancer in regard to morphological tumour characteristics.

Results The study included 61 case of colorectal cancer, assessed by microscopy, immunohistochemistry and descriptive statistical analysis. Regarding cancer grade, there were 45 (73.8% [95% confidence interval (CI) 61.6–83.2]) moderately differentiated (G2) adenocarcinomas, and 16 (26.2% [16.8–38.4]) high-grade (G3) adenocarcinomas. The tumours were mostly advanced, including 27 pT3 (47.3% [32.5–56.7]) and 22 pT4 (36.1% [25.2–48.6]) cancers while pT1 and pT2 cases comprised only 12 (19.8% [11.6–31.3]) tumours. The mean proliferation fraction by Ki-67 was 16.6% [14.9–18.3], and the mean fraction of p53-expressiong neoplastic cells: 11.9% [8.1–15.6]. There were no statistically significant differences between Ki-67 and p53 expression by pT parameters. The mean proliferation fraction (Ki-67) was 15.7% [12.9–18.6] in pT3 and 17.6% [14.8–20.4] in pT4 cancers. The mean fraction of p53 was 11.6% [5.8–17.4] in pT3 and 9.9% [3.9–15.9] in pT4. G2 adenocarcinomas showed expression of Ki-67 in 16.7% [14.7–18.7] of neoplastic cells and p53 in 12.2% [7.5–16.9] of tumour cells, but G3 carcinomas expressed Ki-67 in 16.4% [12.8–20.0] of cells and p53 in 10.1% [3.9–16.2] of neoplastic parenchyma. Spearman rank correlation test showed no significant correlation between p53 and Ki-67 expression (Rs=0.049; p=0.71). By Mann-Whitney test, no statistically significant associations were found between p53 or Ki-67 and tumour grade: p=0.68 and p=0.86, respectively.

Discussion Contrasting with older publications**,** our study did not confirm any differences in mean p53 and Ki-67 expression according tumour grade and pT parameters. This grade- and stage-related homogeneity suggests uniform treatment applicability. Cell proliferation has been associated with better response to neoadjuvant chemotherapy [Lumachi *et al*., 2012] while p53 expression could be targeted by cancer vaccines.

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p53 PROTEIN AS A POTENTIAL TARGET OF CANCER VACCINES IN GLIOBLASTOMAS

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Background: Cancer immunotherapy is aimed at increased specific immunological anti-cancer response by immunization against "cancer specific" proteins. In antitumor vaccines, the selection of the target antigen is among the critical issues. It should be specific to cancer cells and able to activate specific immune response. *TP53* mutations are common in many cancers including glioblastoma (GBM) and can result in high levels of aberrant p53 protein. Thus, p53 antigen seems to be a promising target. Here, we evaluate the expression of p53 protein in GBMs by immunohistochemistry (IHC) to explore whether p53 could become such target in GBMs.

Aims: to evaluate expression of aberrant p53 protein in consecutive GBMs by IHC and computer-assisted morphometry (fraction of positive cells), followed by descriptive statistical analysis including calculation of 95% confidence interval (CI).

Results: The study group comprised 126 patients (63 males and 63 females) who underwent surgery due to morphologically proved GBMs. The mean age of patients was 61.4 [95% CI = 59,4 – 63,4] years. Expression of aberrant p53 protein ranged 0 – 99% of tumour cells, resulting in mean value 35.3% [28.6 – 41.9]. Aberrant p53 protein was absent (0%) from 16 GBMs corresponding to 12.7% [7.9 – 19.7]. Only rare tumour cell nuclei (0 – 5%) expressed p53 in 14 GBMs corresponding to 11.1% [6.6 – 17.9]. However, 40 GBMs, constituting 31.8% [24.2 – 40.3] of the study group, showed strong expression of p53 in >50% of nuclei.

Discussion: Considering the aggressive course of GBMs and limited intervention options, alternative targeted treatments can bring a ray of hope. Vaccines, e.g., anti-EGFRvIII, have induced beneficial immunological and clinical response in GBMs (1). As the direction seems promising, other potential targets should be recognized as well. Here, we have studied p53 pathway by IHC. Although evaluation of *TP53* gene status can be prognostically important, vaccine elaboration necessitates data on protein levels in neoplastic cells. We showed highly variable expression of aberrant p53 protein in GBMs: from complete absence to strong expression in almost all neoplastic cells. Because of inter-tumour heterogeneity, anti-p53 immunotherapy could be effective only in a fraction of GBMs (31.8% in our study). Intra-tumour heterogeneity (reflected by deviations of the expression extremes) indicates the necessity for complex treatment. Although low levels of p53 protein are expressed in normal tissues, immunological response has been reported against p53-overexpressing tumour cells (2). Thus, anti-p53 immunotherapy seems to be possible treatment in p53-overexpressing GBMs. IHC for p53 protein can be suggested as a predictive test.

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EXPRESSION OF ABERRANT p53 PROTEIN IN GASTRIC CANCER

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Background Gastric cancer is one of the most common cancers worldwide (Carcass, 2014). It has complex molecular pathogenesis. The tumour suppressor gene *TP53* has an important role in cell cycle regulation and initiation of tumour genesis (Lazar *et al*., 2010).

Aims The aim of our study is to detect the frequency of p53 expression in gastric cancer by relevant clinical and pathological parameters such as cancer grade and mural invasion.

Results The study group was created by retrospective design, enrolling consecutive potentially radically operated gastric cancer cases (2011 – 2014) from a single university hospital. The resulting group included 103 patients, among them – 66 men (64.1%; 95% confidence interval (CI): 54.5 to 72.7) and 37 women (35.9%; 95% CI: 27.3 to 45.6). Patient's age ranged from 24 to 88 years, mean 67.0 years (95% CI: 57.8 to 76.0). The tumour spectrum by the histological type in accordance to World Health Organisation classification (2010) was following: 83 adenocarcinomas (80.6%; 95% CI: 71.8 to 87.1) and 20 signet ring cell cancers (19.4%; 95% CI: 12.9 to 28.2). The expression of p53 protein was detected by immunohistochemistry. Any case was considered positive if at least 10% of tumour nuclei were positive. In the whole group, p53 expression was found in 48 cases (46.6%; 95% CI: 37.3 to 56.2). The nuclear reactivity was homogenous. Unpaired T test was further applied for statistical analysis and $p<0.05$ was considered significant. Low p53 expression was observed in signet ring cell cancers. Positive expression of p53 was found in 42 (50.6%; 95% CI: 40.1 to 61.1) adenocarcinomas and 6 (30.0%; 95% CI: 14.3 to 52.1) signet ring cell cancers; $p = 0.029$. Regarding the local cancer spread (pT2 *versus* pT3 *versus* pT4), lymph node status (positive *versus* negative for metastases) as well as cancer grade (moderate *versus* high grade), no significant differences were found ($p = 0.705$; $p = 0.117$; $p = 0.314$, respectively). Patient's age and gender did not showed significant differences ($p = 0.459$; $p = 0.352$) in the p53-positive and negative groups.

Discussion The expression of aberrant p53 protein was observed in 46.6% of surgically treatable gastric cancer cases. The signet ring cell cancers showed less frequent p53 protein expression ($p = 0.029$).
The p53 expression is homogeneous by the immunohistochemical distribution. Neither demographic nor such histological data as the local tumour spread, lymph node status or cancer grade showed differences by p53 protein expression suggesting also demographic, grade- and stage-related homogeneity. This makes p53 protein well-suitable for anti-cancer immunization. However, complex treatment must be applied due to intermediate expression rate.

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p53 PROTEIN EXPRESSION IS A RARE EVENT IN PARATHYROID NEOPLASMS

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Background Nowadays, the diagnostic paradigm of primary hyperparathyroidism has shifted significantly from clinically based suspicion in patients presenting with renal or osseous symptoms to almost incidental finding via routine automated serum calcium measurements, parathyroid hormone testing and screening for osteoporosis (DeLellis, 2011). Surgery is increasingly used for the treatment of primary hyperparathyroidism. Thus, molecular studies of parathyroid tissues become possible to find hypothetic future ways of non-surgical treatment (Hong *et al*., 2014).

Aims The aim of this study was to assess the expression of aberrant p53 protein in parathyroid carcinoma and adenoma in comparison to primary parathyroid hyperplasia (PPH) and normal glands.

Results A retrospective analysis of 179 surgically removed parathyroid glands (102 adenomas; 27 PPH; 45 normal glands; 5 carcinomas) was performed. The expression of p53 protein was detected by immunohistochemistry (monoclonal mouse antibody against human p53, clone DO-7, polymeric visualisation system EnVision, Dako, Glostrup, Denmark) and evaluated by computer-assisted morphometry (NIS-Elements/ Eclipse Ci-L, Nikon, Tokyo, Japan). The fraction of p53-positive cells ± standard deviation was 0.92 ± 1.84 (range 0.00 – 7.00; 95% confidence interval (CI): 0.56 – 1.28) in adenoma and 0.08 ± 0.04 (range 0.00 – 0.10; 95% CI: 0.03 – 0.13) in carcinoma. In PPH, the fraction was 2.06 ± 3.61 (range, 0.10 – 11.00; 95% CI: 0.63 – 3.49) and in normal glands 0.02 ± 0.04 (range, 0.00 – 0.10; 95% CI: 0.01 – 0.03). By Kruskal-Wallis test, the differences were statistically significant ($p < 0.001$).

Discussion In parathyroid disease, the p53 protein expression shows statistically significant differences between the researched pathologies. However, the extent of aberrant p53 protein expression is low. Further studies regarding tissue *versus* tumour stem cell differentiation would be necessary both to explain the differences and to assess the hypothetic efficacy of anti-p53 treatment (Hong *et al*., 2014; Zhang *et al*., 2016).

Acknowledgement The research was carried out within the frames of scientific project 08/2013, supported by Riga Stradins University.

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EXPRESSION OF ABERRANT p53 PROTEIN IN HEPATOCELLULAR CARCINOMA

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Background Hepatocellular carcinoma is one of the most common malignancies worldwide. The major risk factors include chronic infections with the hepatitis B or C virus. Multiple genetic and epigenetic changes are involved in the molecular pathogenesis of hepatocellular carcinoma, including somatic mutations in the p53 tumour suppressor gene *TP53* [1]. Since the p53 gene is frequently mutated or inactivated in different cancer types, it is a highly attractive therapeutic target for treating the disease. The success of targeting p53 is likely to depend on the frequency of pathological aberrations and availability of a predictive biomarker [2].

Aim To evaluate p53 protein expression in hepatocellular carcinoma.

Results In a retrospective study, 35 cases of morphologically confirmed hepatocellular carcinoma were included, based on the tissue availability in liver biopsy or resection material. p53 protein expression was evaluated by immunohistochemistry and assessed quantitatively by computer-assisted morphometry as the fraction (%) of positive neoplastic cells. Only intense nuclear reactivity was considered positive. The hepatocellular carcinoma showed p53 expression in 16 (45.7%; 95% confidence interval (CI) = 30.5 – 61.8) out of 35 cases, of which 11 (68.6%; CI = 44.4 – 85.8) had only 30% or less positive cells.

Discussion In many cases tumour antigen-specific vaccination has been perceived as a potentially effective approach to improve patients' outcome by mobilizing anti-tumour immunity. As known, the selection of a target antigen is one of the most critical issues in anti-tumour vaccines. In this study we evaluated p53 protein expression in hepatocellular carcinoma and concluded that expression of p53 in this type of primary liver tumours is limited both by frequency and by extent. As p53 peptide vaccine may elicit an HLA-A2.1-restricted cytotoxic T lymphocyte immune response against tumour cells that overexpress p53 protein [3], either different or combined treatment options should be considered regarding hepatocellular carcinoma.

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EXPRESSION OF p53 PROTEIN IN LUNG CANCER

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Background Lung cancer is clinically, morphologically and molecularly heterogeneous disease. The main prognostic factor is the stage of the tumour depending on TNM classification (Travis *et al*., 2015). Nevertheless, valuable data are provided by the tumour molecular characteristics, especially the alteration of apoptosis, expression of the transcription factor and tumour suppressor p53 protein that has a potential of becoming a target for novel treatment options (Uramoto *et al*., 2006).

Aim To evaluate p53 protein expression in primary lung adenocarcinoma, squamous cell carcinoma and small cell carcinoma.

Results Retrospective study included 30 consecutive cases of primary lung adenocarcinoma, squamous cell carcinoma and small cell carcinoma each that were acquired from the lung biopsy and pulmonary resection material. p53 protein expression was evaluated by immunohistochemistry and assessed by computer-assisted morphometry in tumour areas with the highest marker expression. The p53 was regarded positive if intense nuclear staining was found in 10% or more of the tumour cells.
A total of 29 (96.7%; 95% confidence interval (CI) = 83.3 – 99.4) cases of adenocarcinoma expressed p53, and in 23 tumours (76.7%; CI = 59.1 – 88.2) the protein was detected in more than 10% of the neoplastic cells.
The squamous cell carcinoma showed p53 expression in 25 (83.3%; CI = 66.4 – 92.7) out of 30 cases of which 22 (73.3%; CI = 55.6 – 85.8) had 10% and more positive cells.
Furthermore, in small cell carcinoma the protein expression was detected in 22 (73.3%; CI = 55.6 – 85.8) cases, while expression exceeded the set threshold level of 10% in 20 (66.7%; CI = 48.8 – 80.8) of these tumours.

Discussion Expression of p53 protein can be observed in all the most frequent lung cancer types, namely, in primary pulmonary adenocarcinoma, as well as pulmonary squamous cell and small cell carcinoma, in significant frequency and extent. These findings could be considered important when novel therapeutic treatments of lung cancer will emerge, e.g., small molecular weight compounds restoring the activity of p53 pathway or anti-p53 vaccines (Hong *et al*., 2014).

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EXPRESSION OF ABERRANT p53 PROTEIN IN MEDULLOBLASTOMA

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Background Medulloblastoma, a highly malignant embryonal neuroepithelial neoplasm, classified as grade IV tumour by World Health Organisation (Louis *et al*., 2007) comprises 20% of childhood brain tumours. Although major advances in oncology have resulted in increased 5-year survival reaching 55 – 80%, further improvements are necessary, including also treatment options that would induce less side- and residual effects. As p53 pathway has recently appeared as an attractive treatment target (Zhang *et al*., 2016), we evaluated medulloblastomas for the accumulation of aberrant p53 protein suggesting a subclass of *TP53* gene mutations.

Aims The aim of the present study was to evaluate the frequency and extent of pathological p53 protein accumulation in medulloblastoma tissues.

Results The study was designed by retrospective approach. All consecutive cases of medulloblastoma were retrieved from archives of a single university hospital providing tertiary care for childhood diseases in Latvia. Within the time period of 2000 – 2016, there were 26 verified cases including 20 (76.9%; 95% confidence interval (CI): 57.6 – 89.3) boys and 6 (23.1%; 95% CI: 10.7 – 42.4) girls. The mean age was 79.2 months (standard deviation (SD) 42.9; 95% CI: 62.6 – 95.60), ranging 12 – 160 months. Tissues were available in 20 cases, including 14 (70.0%; 95% CI: 47.9 – 85.7] boys and 6 (30.0%; 95% CI: 14.3 – 52.1] girls. The mean age in this subgroup was 81.6 months (SD 44.7; 95% CI: 62.0 – 101.1), and the age ranged 16 – 160 months. Thus, the immunohistochemically evaluated cases showed no statistically significant demographic differences from the whole group. p53 protein was detected by immunohistochemistry and evaluated by morphometry to identify the fraction of neoplastic cells (%) exhibiting intense nuclear reactivity. The fraction of p53-expressing cells ranged from a completely negative finding (0.0%) in a single case (5.0%; 95% CI: 0.0 – 25.4) to 52.4%; the mean value was 11.7% (SD 12.2; 95% CI: 6.4 – 17.1). In 8 cases (40.0%; 95% CI: 21.8 – 61.4), the fraction of p53-positive cells exceeded 10%. There was no significant correlation with proliferation fraction by Ki-67 (Pearson test; $p = 0.93$).

Discussion Although most cases of medulloblastoma express aberrant p53 protein, only a fraction of tumour cells is positive. Thus, p53-targeted treatment (Zhang *et al*., 2016), including anti-p53 cancer vaccines, could be helpful in a combined approach. Further studies would be necessary to evaluate the relation between expression of aberrant p53 and cancer stem cells (Hong *et al*., 2014) in medulloblastoma as close association between these features would suggest higher anti-p53 treatment efficacy. In addition, the specific immunologic traits of central nervous system must be encountered.

Acknowledgement The research was carried out within the frames of scientific project 08/2013, supported by Riga Stradins University.

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Day 2, May 25, 2016

SESSION IV: OVERVIEW OF THE CANCER FIELD

Chairs: Birke Bartosch, Britta Wahren

9.00 - 09.40 Manuel Patarroyo (Karolinska Institutet, Stockholm, Sweden) Introductory lecture on cancer development and vaccine targets. Laminin isoforms in tumor invasion and metastasis.

09.40 - 10.20 Ann-Kristin Östlund Farrants (Stockholm University, Stockholm, Sweden) Chromatin remodeling and cancer, the role of factors of chromatin remodeling in carcinogenesis.

10.20 - 10.35 Rainer Schindl (JKU University of Linz, Austria) Activation of transcription factors by carcinogenic Orai mutants.

SESSION IV: OVERVIEW OF THE CANCER FIELD (continued)

Chairs: Manuel Patarroyo, Lars Frelin

11.00 - 11.30 Alexander Ivanov (Engelhardt Institute of Molecular Biology, Moscow, Russia) Oxidative stress and carcinogenesis.

11.30 - 12.10 Birke Bartosch (INSERM, Lyon, France) Metabolic reprogramming - a hallmark of oncogenic viruses.

12.10 - 12. 25 Bhupesh Prusty (University of Würzburg, Würzburg, Germany) Understanding ciHHV-6 reactivation through miRNAs and mitochondrial network: a new prospective towards better therapy.

12.25 - 12.40 Muhammad Mushtaq (Karolinska Institutet, Stockholm, Sweden) S18 family of mitochondrial ribosomal proteins: evolutionary history and gly132 polymorphism in colon carcinoma.

SESSION V: TARGETS OF CANCER VACCINES

Chairs: Elena Kashuba, Dace Pjanova

14.00 - 14.20 Karaman Olga (Institute of Experimental Oncology and Radiology, Kiev, Ukraine) Vaccines in cancer immunotherapy: experience of Ukrainian oncologists

14.40 - 15.20 Jon Amund Kyte (The Norwegian Radium Hospital, Oslo, Norway) Telomerase reverse transcriptase as a target of anticancer vaccines.

15.20 - 15.40 Britta Wahren (Karolinska Institutet, Stockholm, Sweden) Immunotherapeutic targeting of carcinoembryonic antigen.

SESSION V: TARGETS OF CANCER VACCINES (continued)

Chairs: Dzmitry Shcharbin, Irina Kholodnyuk

16.00 - 16.40 Galina Selivanova (Karolinska Institutet , Stockholm, Sweden) Mutant p53 vaccination prospects.

16.40 - 17.20 Elena Kashuba (Institute of Experimental Oncology and Radiology, Kiev, Ukraine) Mitochondrial protein S18-2: functions and possibility of vaccine targeting.

17.20 - 17.30 Monta Ustinova (Riga Stradins University, Riga, Latvia) Impact of low penetrance variants on breast cancer morbidity and prognosis.

LAMININ ISOFORMS IN TUMOR INVASION AND METASTASIS

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Tumor invasion and metastasis account for nearly 90% of all cancer-related deaths, and nearly 10 million people die from cancer every year. Laminins, a family of extracellular matrix proteins mainly found in basement membranes, are masters of tissue architecture, a property which is highly deregulated during tumor invasion and metastasis. Over 16 laminin isoforms are presently known and their expression is developmentally regulated and cell- and tissue specific. Laminins are recognized by integrins and other cell-surface receptors and promote cell adhesion, migration, survival, stemness and proliferation, cellular processes involved in the metastatic cascade. Although expression of laminin isoforms in tumors mostly reflects expression in their normal counterparts, distinct alterations of laminin expression and function occur during tumor invasion, particularly in epithelial-to-mesenchymal transition of the tumors cells and loss of the basement membrane barrier. During dissemination and metastasis cancer cells encounter vascular, neural, lymphoid tissue and other exogenous laminins. However, the malignant cells themselves are able to produce and secrete laminins and to use these endogenous molecules in an autocrine fashion. Recent studies have demonstrated participation of tumor cell-derived laminins in different steps of the metastatic cascade. RNA interference of particular tumor cell laminins inhibits tumor invasion and metastasis and mouse monoclonal antibodies to the laminins inhibit tumor cell migration and renewal. Expression of malignancy-associated laminins is much higher in malignant cells than in pre-malignant cells, and overexpression of these laminins in tumors significantly correlates with reduced patient survival. The contribution of tumor cell laminins to various steps of the metastatic cascade, such as tumor cell migration, survival, self-renewal and proliferation, will be presented, as well as the regulation of these laminins by oncogenic pathways and their potential as therapeutic targets.

The present study was supported by Cancerfonden and Karolinska Institutet.

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CHROMATIN REMODELING AND CANCER, THE ROLE OF FACTORS OF CHROMATIN REMODELING IN CARCINOGENESIS

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Background The chromatin structure is a major regulator of nuclear processes and at the same time it protects the DNA from DNA damage and DNA breaks. Several proteins regulating the compaction of chromatin have now emerged as being dysfunctional in cancer, playing a role in cancer initiation and progression. In particular, erroneous alternations in the chromatin structure leads dysregulation of gene expression and to loss of genomic stability. Chromatin is also involved in fundamental processes, such as such as ribosomal biogenesis, which constitutes a major part of any cells activity. These genes are present in multiple copies in human cells, but not all gene copies are actively transcribed in differentiated cells. Whether genes are active or silent is determined by the chromatin structure at the individual genes; more open at active genes and heterochromatic at silent genes. In addition to provide the cells with rRNA for ribosome biogenesis, the ribosomal transcription acts as a stress sensor; in response to transcriptional stress, DNA damage, heat shock and hypoxia, transcription is stopped. Because of the repetitive nature of the genes, they are also prone to recombine and the heavy transcription constitutes a danger of transcription mediated DNA damage, potentially causing genetic instability.

Aims Chromatin remodelling factors in the SWI/SNF complex has been mutated and deleted in a variety of tumours, resulting in an altered expression pattern and maybe splicing pattern. Fundamental processes, such as the ribosomal transcription is also often dysregulated in cancer cells, by overexpression of oncogene regulating the transcription and by overexpression of RNA polymerase factors. The prevailing idea in the field is that the dysregulation of the ribosomal transcription is required to meet the need for more ribosomes in highly proliferating cancer cells. However, some tumour cells have a reduced transcription, not showing a need for more ribosomes. Despite the lower transcription rate, tumour cells are more sensitive to different drugs and stress factors suggesting that the malignant transformation has an impact on the ribosomal genes in other ways than only to alter the transcription rate. Here, we assess the role of chromatin changes at the rDNA loci in malignant transformation.

Results and Discussion: Several chromatin remodelling factors and histone modifying factors are involved in the regulation of the chromatin at rDNA. We have isolated an ATP dependent chromatin remodelling complex, B-WICH, comprised of William syndrome transcription factor (WSTF), the ATPase SNF2h and nuclear myosin, which are required for transcription. We propose that this is an early activator needed for cells to respond to environmental stimuli. The WSTF are haploinsuffiently expressed in William Syndrome and some features are most likely caused by ribosomal transcription defect, classifying William Syndrome as a ribosomopathy. Recently, several other ribosomopathies, such as Diamond-Blackfan anaemia and Schwachmann-Diamond anaemia, affecting haematopoietic development also predispose these individuals to leukemia. William syndrome patients are not more prone to develop cancer, however, WSTF is associated with cancer when overexpressed. The question arise whether dysregulation of transcription factors of rRNA genes results in a changed chromatin structure of the rDNA, leading to genetic instability.

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ACTIVATION OF TRANSCRIPTION FACTORS BY CARCINOGENIC ORAI MUTANTS

Romana Schober, Monika Litvinukova, Irene Frischauf, Christoph Romanin, Ivan Bogeski and Rainer Schindl

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Background The store-operated Ca^{2+} channel components STIM1 and Orai1 are important for T cell activation and mast cell degranulation (1) but pathophysiological STIM1/Orai1 Ca^{2+} signaling has been shown to contribute to critical steps in malignant cancer cell development (2).

Aims Here we aimed to analyze Orai1 point mutants, determined from large genome wide cancer studies, for pathophysiological cell functions including constitutive calcium influx and the activation of calcium-regulated transcription factors.

Results We analyzed these Orai1 cancer mutants upon overexpression in HEK cells for constitutive Ca^{2+} influx and activation of the nuclear factor of activated T-cells (NFAT), transcription factor EB (TFEB) and microphthalmia-associated transcription factor (MITF). We found that five Orai1 cancer mutants induced significant increased MITF translocation to the nucleus and one Orai1 mutant which lead to increased TFEB activation in the absence of STIM1 and store-depletion. In confocal fluorescence microscopy, we studied the time-dependent translocation from the cytosol to the nucleus for NFAT in comparison to MITF and TFEB upon co-expression of STIM1 and Orai1 by thapsigargin. Only NFAT translocated quickly upon store-depletion in contrast to the other transcription factors, suggesting different Ca^{2+} dependent processes. NFAT translocation mediated by constitutively active Orai1 mutants is induced by activation of the Ca^{2+} dependent phosphatase calcineurin.

Discussion Out of 20 published Orai1 mutants found in cancer patients, five Orai1 mutants induced significant increased MITF nuclear translocation and one Orai1 mutant lead to increased TFEB activation in the absence of additional cell stimulus. Further, we will discuss a potential role of calcineurin in the activation process of MITF and TFEB.

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OXIDATIVE STRESS AND CARCINOGENESIS

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Background Cancers represent one of the leading causes of human morbidity and mortality worldwide. The World Health Organization expects cancer rates to rise by up to 70% in the next few years [1]. Recent progress has improved treatment outcome for most cancers, however for some cancer types and in particular for lung, liver, and brain cancer cure rates remain low and prognosis very poor. Therefore, investigation of the molecular mechanisms underlying malignant transformation in these types of cancer and cancer in general is of great importance. Carcinogenesis is a multistep process that involves genomic instability and accumulation of mutations, dysregulation of cell signaling pathways, long-lasting inflammation, and metabolic adaptation. These events can be triggered by a variety of factors including environmental factors, infections, and sporadic genetic alterations depending on a cancer type. Importantly, almost all these events include dysregulation of the cellular redox system.

Aims Here we present a systematic analysis of data evidencing importance of reactive oxygen species (ROS) in development of liver cancer by hepatitis B and C viruses (HBV, HCV).

Results Hepatitis B and C viruses frequently induce inflammatory processes that trigger chronic liver disease characterized by fibrosis and on the long term cirrhosis and hepatocellular carcinoma. Noteworthy, both viruses trigger massive ROS production *in vitro* and *in vivo*. Oxidative stress occurs through activation of multiple ROS-generating enzymes/systems and dysregulation of ROS-scavenging systems of the host cells. Markers of oxidative stress in CHB/CHC patients strongly correlate with severity of liver disease and development of fibrosis or cancer. Increased levels of ROS contribute to production of proinflammatory and profibrotic cytokines in the infected hepatocytes. They also participate in the dysregulation of cell signaling pathways and provide a significant insult for the host genome. Finally, ROS-producing and ROS-scavenging systems are tightly linked to cellular metabolic pathways including metabolism of glucose and glutamine, lipids, and biogenic polyamines.

Discussion Thus, oxidative stress represents a complex event that plays a key role in the development of many types of cancer including hepatocellular carcinoma. Its detailed investigation may provide better understanding of molecular mechanisms of carcinogenesis and provide clues to development of novel treatment or prevention strategies.

Acknowledgements The authors were supported by Russian Ministry of Education and Science (Agreement №14.616.21.0043) (A.I.) and PHC Kholmogorov (B.B.).

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METABOLIC REPROGRAMMING - A HALLMARK OF ONCOGENIC VIRUSES

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More than one in ten cases of cancer in the world are due to chronic viral infections. Viruses induce oncogenesis by targeting the same pathways known to be responsible for neoplasia in tumor cells, such as control of cell cycle progression, cell migration, proliferation and evasion from cell death and the host's immune defense. In addition, metabolic reprogramming, characterized by activation of biosynthetic pathways has been identified over a century ago as a requirement for growth of transformed cells in order to provide sufficient levels of energy and building blocks for proliferation. Interestingly, viruses introduce into their host cells similar metabolic adaptations, and importantly, it seems that they depend on these changes for their persistence and amplification. The central carbon metabolism, for example is not only frequently altered in tumor cells but also modulated by human papillomavirus, Epstein-Barr virus, Kaposi's Sarcoma-associated virus and hepatitis B and C viruses. For example, metabolic adaptations induced by hepatitis C virus (HCV), a major cause of hepatocellular carcinoma, target in particular glycolysis and glutaminolysis, metabolic pathways known to play an important role in neoplastic transformation as they ensure the balance between cellular energetics, biosynthesis and stress defense. While HCV induces glutaminolysis to create an environment favorable for viral replication, it predisposes infected cells to transformation. Thus glutaminolytic but also glycolytic enzymes are emerging as interesting therapeutic targets for prevention of hepatocarcinogenesis in the context of chronic hepatitis, but potentially also in the context of infection with other oncogenic viruses.

UNDERSTANDING ciHHV-6 ACTIVATION THROUGH miRNAS AND MITOCHONDRIAL NETWORK: A NEW PROSPECTIVE TOWARDS BETTER THERAPY

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Background: The innate immune system is the first line of host defense against viral or bacterial invasions and is normally triggered by the recognition of pathogen-associated molecular patterns (PAMPs). Double-stranded RNA (dsRNA) of RNA viruses is recognized by TLR-3 or either of the two retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), RIG-I and MDA-5 (melanoma differentiation-associated gene 5) initiating distinct signaling pathways. However, in an indirect way DNA-viruses like human herpesviruses are also recognized by RIG-I and MDA5. Intriguingly, mitochondria constitute an important platform for several adapter molecules during antiviral signaling. Many viruses target the cellular antiviral pathway of the innate immune system to inhibit antiviral response by either inhibiting or inducing mitochondrial functions.

Aims: We are curious to understand the survival stratergies of one of the most successful infectious organisms, Human herpesvirus-6 (HHV-6), having a seroprevalence of almost 100% in the human population.

Results: Our study show that HHV-6 viral DNA is directly recognized by host cell innate immune system through activation of RIG-I/MAVS signaling pathway. We also show that HHV-6 infection/activation efficiently down regulates one of the host miRNA family members, miR30, within hours of infection leading to p53 and Drp1 upregulation which induces a change is host cell mitochondrial fusion-fission dynamics thereby favoring mitochondrial fragmentation. Using stable cell lines expressing soluble GFP within mitochondria and super resolution microscopy, we show that mitochondrial fragmentation is necessary for viral survival inside the host cell and is caused by recruitment of Drp1 onto the mitochondrial fission rings. As heat or UV-inactivated HHV-6 particles could not down regulate viral DNA-induced RIG-I and MAVS expression, we believe that an immediate early HHV-6 protein might be necessary for miR30 down-regulation. Using various human cell types containing chromosomally integrated HHV-6, we provide evidence for a unique type of viral activation that does not lead to viral DNA replication and complete viral particle formation.

Discussion:
As miR30 down regulation is not observed in cells allowing latent viral infection, we argue that miR30 down regulation might be the key step in viral life cycle that decides the balance between lytic and latent viral infection. Our work also raises concerns regarding the use current antiviral drugs to treat HHV-6 activation in the absence of viral DNA replication.

S18 FAMILY OF MITOCHONDRIAL RIBOSOMAL PROTEINS: EVOLUTIONARY HISTORY AND GLY132 POLYMORPHISM IN COLON CARCINOMA

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Background: S18 family of mitochondrial ribosomal proteins (MRPS18, S18) consists of three members, S18-1 to -3. Earlier, we found that overexpression of S18-2 protein resulted in immortalization and eventual transformation of primary rat fibroblasts. The S18-1 and -3 have not exhibited such abilities. To understand the differences in protein properties, the evolutionary history of S18 family was analyzed.

Aims: The present study is devoted to two aims: (i) to gain insight into the evolution of S18 protein family and (ii) to create a link between the knowledge obtained from evolutionary analysis of S18 proteins and cancer development in humans. Here we focused on origins, evolutionary patterns and phylogeny of S18 family proteins in eukaryotic and bacterial clades, and also analyzed the mutations of evolutionarily conserved residues in different types of cancer.

Results: The S18-3, followed by S18-1 and S18-2 emerged as a result of ancient gene duplication in the root of eukaryotic species tree, followed by two metazoan-specific gene duplications. However, the most conserved metazoan S18 homolog is the S18-1: it shares the most sequence similarity with S18 proteins of bacteria and of other eukaryotic clades. Evolutionarily conserved residues of S18 proteins were analyzed in various cancers. S18-2 is mutated at a higher rate, compared with S18-1 and -3 proteins. Moreover, the evolutionarily conserved residue, Gly132 of S18-2, shows genetic polymorphism in colon adenocarcinomas that was confirmed by direct DNA sequencing.

Discussion: Two phylogenetic gene trees were constructed for eukaryotic S18 and bacterial S18 proteins. Eukaryotic S18 gene tree was further analyzed and reconciled with species tree, using MPR to infer the taxonomic branches, where probable duplications occurred and three eukaryotic S18 homologs emerged. Evolutionary trace analysis was then performed to infer the most conserved residues and place on the protein structure, where these residues can be found.
Finally, the mutational status of evolutionarily conserved residues in S18 family proteins was analyzed in different types of cancer. We found an interesting genetic polymorphism in S18-2 that might be an important biomarker for colon adenocarcinoma.

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VACCINES IN CANCER IMMUNOTHERAPY: EXPERIENCE OF UKRAINIAN ONCOLOGISTS

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Background Cancer vaccines were used by WB Coley more than a century ago [1]. The progress in molecular technology has allowed to discover a number of tumor antigens and tumor-associated complexes which could serve as potential targets for effectors of antitumor immunity. In parallel, technologies of cancer vaccine preparation have been developed as well. The high efficacy of cancer vaccines prepared from whole tumor cells or their lysates was shown [2]. In Ukraine, the development of cancer vaccines and research of cancer immunotherapy have been initiated by prof. D.G. Zatula [3] and were continued in R.E. Kavetsky IEPOR NAS of Ukraine. The original technology of preparation of cancer vaccine from autologous tumor material modified with cytotoxic lectin of *B. subtilis* B-7025 has been developed.

Aim To perform a comprehensive analysis of the results of experimental and clinical studies of cancer immunotherapy with the use of autovaccine (CAV) developed at IEPOR NASU.

Results *In vivo* studies on different experimental tumor models (including metastatic Lewis lung carcinoma) have shown that CAV-based immunotherapy resulted in suppression of primary tumor growth and significant antimetastatic effect if the primary tumor is surgically removed. The CAV-based vaccination potently affects the state of the immune system, in particular, it activats cellular and humoral effector responses of adaptive immunity and also nonspecific antitumor resistance. The production of IL-2, TNF and IL-1 by immune cells *in vitro* was increased. Probably, CAV could act via the prolongation of destructive phase of macrophage activation and preservation of functional reserve of these cells as well as the slowing of tumor-related immunosuppression.

The promising results of preclinical studies have formed the basis for the clinical trials. Here CAVs have been used after surgical treatment of cancer patients (adjuvant administration); immunotherapy was included into the standard chemotherapy and radiotherapy protocols of the patients (according to their clinical diagnosis). The CAV efficacy was evaluated by comparison of relapse–free survival and overall 1-year and 5-year survival in patients treated by convenient therapy versus those who additionally received the immunotherapy. CAV-based immunotherapy was performed in patients with colorectal, gastric, lung, breast, kidney cancer and malignant brain tumors, and has demonstrated significant long-term results. Immunological effects of CAV administration were similar in the clinical trials and experimental studies. The CAV efficiency was shown to be dependent on histological type of the tumor, clinical stage of cancer process, and tumor grade.

Conclusions The experimental and clinical studies of the Ukrainian oncologists evidence on an expediency of inclusion of cancer autovaccines into regimens of cancer therapy for extending of patients' life and improvement of its quality.

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TELOMERASE REVERSE TRANSCRIPTASE AS A TARGET OF ANTI-CANCER VACCINES

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Background: Telomerase is highly expressed in most cancer forms, while the expression in normal tissues is restricted. We have conducted a series of clinical trials evaluating vaccine therapy with the telomerase peptide GV1001, in patients with melanoma, non-small cell lung cancer (NSCLC) or pancreatic cancer[1-3]. The studies demonstrated no serious side effects, immune responses in 50-80% of patients and an improved survival for immune responders, compared to non-responders.

Aims: We noted considerable differences in clinical outcome among immune responders in the GV1001 vaccine trials, highlighting that an immune response is not necessarily beneficial. To investigate what characterizes the effective immune response, we conducted a follow-up study in NSCLC patients vaccinated with GV1001. We performed long term clinical monitoring and studied the immune response with regard to factors hypothesized to be important for clinical efficacy; myeloid suppressor cells, T regulatory cells and cytokine profile. Peripheral blood mononuclear cells from 33 NSCLC trial patients and 15 healthy donors were analyzed by flow cytometry for T regulatory cells (Tregs; $CD4^+CD25^+CD127^{low/-}FOXP3^+$) and two types of myeloid derived suppressor cells (MDSCs; *HLA-*$DR^{low}CD14^+$ *or* $Lin^{-/lo}$*HLA-*$DR^-CD33^+CD11b^+$). T cell cultures were analyzed for 17 cytokines

Results Immune responders had increased overall survival (OS; $p<0.001$) and progression free survival (PFS; $p=0.003$), compared to subjects without immunological response. The mean OS advantage was 54 versus 13 months. Six patients were still alive at the last clinical update, all belonging to the immune responders. No serious toxicity had developed (maximum observation 13 years). Most patients developed a polyfunctional cytokine profile, with high IFN-g/IL4 and IFN-g/IL-10 ratios. Low Treg levels were associated with improved OS ($p=0.037$) and a favorable cytokine profile, including higher IFN /IL-10 ratios. High CD33+ MDSC levels were associated with poorer immune response rate ($p=0.005$). The levels of $CD14^+$ MDSC were significantly higher in patients than healthy controls ($p=0.012$).

Discussion We consider that a randomized GV1001 trial in NSCLC or malignant melanoma is warranted, based on a high immune response rate, favorable cytokine profiles, low toxicity after long term observation and superior survival for immune responders. The findings in the present NSCLC study suggest that Tregs and MDSCs are associated with a tolerogenic cytokine milieu and impaired clinical efficacy of vaccine responses. The data also indicate that HLA-$DR^{low}CD14^+$ MDSCs are associated with development of NSCLC.

Acknowledgements The work was supported by the Norwegian Health Region South-East and the Faculty of Medicine, University of Oslo.

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IMMUNOTHERAPEUTIC TARGETING OF CARCINOEMBRYONIC ANTIGEN

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Background: Colorectal carcinoma is a leading cause of cancer-related mortality. Despite introduction of new drugs, a large proportion of patients remain incurable. Plasmid DNA, encoding tumor antigens such as carcinoembryonic antigen (CEA), represents a novel approach of delivering conformational antigens.

Aim: Immunization by CEA-DNA constructs induces antigen-specific humoral and cellular responses experimentally and in the clinic. Our intention was to improve on earlier CEA glycoprotein immunogenicity found in a phase 1 therapeutic study (1). CEA-DNA plasmids were administered by needle-free Bioject or by electroporation.

Results: We report immune data of an explorative study using CEA66-DNA (non-glycosylated CEA) and tetwtCEA-DNA (glycosylated CEA) for immunization in combination with cyclophosphamide and GM-CSF in the clinical adjuvant setting of colo-rectal cancer (2, 3). A significant increase in antigen-specific CD4+ effector memory, CD8+ effector and CD8+ effector memory T cells was found after repeated CEA-DNA immunization with the non-glycosylated CEA-DNA construct given by needle-free Biojector.

Discussion: Both delivery methods of DNA plasmids were tolerable and safe. The induction of CEA-specific T-cell responses indicated reduced immunological tolerance particularly by the non-glycosylated CEA66-DNA immunogen. CEA-DNA delivery by Biojector seemed to be as good as electroporation for induction of cell.mediated immune responses. Since previous immunization by CEA glycoprotein had evoked antibody responses related to prolonged time to recurrence, it seems prudent to combine DNA with protein (or viral vectors) to obtain both humoral and cellular immune responses in the usually immunosuppressed cancer patient.

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MUTANT p53 VACCINATION PROSPECTS

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p53 is a potent tumor suppressor which prevents cancer development by eliminating cells carrying oncogenic mutations inducing apoptosis, growth arrest or senescence. Therefore is it not surprising that p53 inactivation by point mutation is a common feature of around 50% of human cancers. Depending on a type of a tumor, such as for example ovarian carcinoma and small cell lung cancer, the frequency of p53 mutations can reach 90%. Mutant p53 proteins, in addition to loss of the tumor suppressor function, gain oncogenic activities, contributing to the aggressiveness of cancer. Since mutant p53 proteins can not induce p53's own E3 ligase MDM2, the stability of mutant p53 proteins in increased resulting in their accumulation in cancer cells. High levels of mutant p53 are immunogenic, as is evidenced by the presence of anti-p53 antibodies in patients with mutant p53-carrying cancers. Furthermore, at least some mutations, such as the p53 Y220C mutation can be processed and presented for CD8+ T cell recognition.

In conclusion, peptides derived from mutant p53 protein could be regarded as important tumour-associated antigens that can be used for the development of anti-cancer vaccines.

IS MRPS18-2 THE NEW ONCOGENE OR A MASTER REGULATOR OF CELL DIFFERENTIATION?

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Background: We have found that mitochondrial ribosomal protein S18-2 (MRPS18-2) is involved in regulation of the RB-dependent pathway. It binds to both, hypo- and hyper-phosphorylated RB protein. The binding between RB and S18-2 proteins is promoted when cytoplasmic S18-2 is targeted to the nucleus, and this disrupts the association of E2F1 with RB, as indicated by the increased level of free E2F1 in the nucleus. This presumably lifts the RB-dependent block to S-phase entry in the cell cycle. We have also found that overexpression of the human S18-2 immortalized primary rat embryonic fibroblasts and they showed properties of embryonic stem cells (1). Terminally differentiated skin fibroblasts were transformed upon S18-2 overexpression (2). Moreover, S18-2 increased in endometrial cancers compared with the normal endometrium and hyperplasia, based on a study of 42 patient biopsies (3).

Aims: Elevated expression of S18-2 in stem and tumor cells (our findings and analysis of published microarray data) raises the question of whether this protein co-operates with the RB protein in differentiation and cancerogenesis. In the present work we aimed to characterize the pathways of cell fate regulation with the involvement of S18-2 and RB proteins.

Results: We showed that S18-2 protein, together with RB, plays a crucial role in cell de-differentiation. We have found that overexpression of S18-2 and RB is needed for maintenance of cell stemness. Such cells can differentiate into various cell lineages under certain conditions.

Discussion: We have shown that S18-2 could enhance the telomerase activity. Overexpression of S18-2 induced chromosomal instability in the transfected cells. Concluding, the s18-2 is a novel oncoprotein that also is involved in control on cell stemness.

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IMPACT OF LOW PENETRANCE VARIANTS ON BREAST CANCER MORBIDITY AND PROGNOSIS

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Introduction: The impact of high-penetrance gene variants on breast cancer risk is widely described, nevertheless they account for only about 25% of the familial risk and less than 5% of total breast cancer predisposition [1]. It is suggested that remaining risk may result from a multiplicative effect of common low-penetrance gene variants [2]. Therefore low-penetrance gene variants and their combinations are topical study objects in breast cancer pathogenesis [3]. Four of low-penetrance variants were chosen to test their impact on breast cancer morbidity and prognosis in the given population.

Aim: To determine the impact of four low-penetrance gene variants on breast cancer morbidity and prognosis.

Materials and Methods: Study group consisted of 2,530 European descent breast cancer patients without proven founder variants c.181T>G (p.Cys61Gly), c.4035delA (p.Glu1346Lysfs) and c.5266dupC (p.Gln1756Profs) in the *BRCA1* gene (MIM 113705) and/or positive family history of disease, indicating a possible presence of high penetrance variant. 731 European descent voluntary women with no oncologic illnesses reported at the time of application were included in the control group. SNP genotyping was carried out by restriction fragment length polymorphism and TaqMan SNP genotyping assays. Data analysis was performed using the program Rv3.1.0.

Results: Case-control study results are presented below. Notably protective effect was

SNP	Zygosity	OR	95% CI	P-value
rs9693444	Heterozygous	1.39	1.11-1.75	0.005
	Homozygous	1.51	1-2.34	
rs1436904	Heterozygous	0.79	0.63-1	0.008
	Homozygous	0.62	0.45-0.87	
rs616488	Heterozygous	0.86	0.68-1.09	0.367
	Homozygous	0.84	0.57-1.25	
rs204247	Heterozygous	0.88	0.68-1.14	0.423
	Homozygous	1.04	0.75-1.45	

observed in the case of rs1436904 positive and rs9693444 wild type (wt) genotype compared to rs1436904wt and rs9693444 positive allele combination (OR=0.54; 95% CI=0.39-0.73; p<0.001). Disease specific survival rates did not show significant prognostic impact for any of the variants tested. Worse prognosis trend (p=0.0877) was observed for rs9693444 positive and rs1436904wt genotype compared to rs1436904 positive and rs9693444wt genotype.

Discussion: The results of our study suggest that low-penetrance gene variants have a multiplicative effect on breast cancer morbidity. They also imply the necessity for extended study with increased number of individuals in study groups and additional allelic variants.

Conclusion: Rs9693444 proved association with increased breast cancer risk, rs9693444 has a protective effect, but rs616488 and rs204247 has no impact on breast cancer risk in the given population. Funded by National Research Program BIOMEDICINE for Public Health.

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Day 3, May 26, 2016

SESSION VI: CANCER VIROTHERAPY

Chairs: Lars Frelin, Jon Amund Kyte

09.00 - 09.40 Dace Pjanova (Biomedical Research and Study Center, Riga, Latvia) Immunotherapy of melanoma.

09.40 - 10.00 Dace Reihmane (Riga Stradins University, Riga, Latvia) Cancer immunotherapy: oncolytic virotherapy in Latvia.

10.00 - 10.40 Marion Schneider (University Hospital Ulm, Ulm, Germany) NK mediated cytolysis in glioblastoma, triggered by viral therapy.

10.40 - 10.45 Poster presentation (5 min; 3-5 slides) Telle V, Patetko L, Tilgase A, Ramata Stunda A, Borodušķis M, Alberts P. Effect of Rigvir on viable cancer cell count *in vitro*.

SESSION VII: DESIGN AND DELIVERY OF VACCINES AND BIOPHARMACEUTICALS

Chairs: Dzmitry Scharbin, Karl Ljungberg

11.10 - 11.50 Elżbieta Pedziwiatr-Werbicka (University of Lodz, Lodz, Poland) Nanoparticles as the basis for treatment of HIV/AIDS and cancer.

11.50 - 12.30 Joel Palefsky (University of California, San Francisco, USA) Immunotherapy for HPV-related disease in HIV-positive and HIV-negative men and women.

12.30 - 13.10 Elizaveta Starodubova (Chumakov Institute of Poliomyelitis and Viral Encephalitides, Engelhard Institute of Molecular Biology, Moscow, Russia, and Karolinska Institutet, Stockholm, Sweden) Breaking through low immunogenicity of HIV-1 reverse transcriptase in therapeutic vaccines against drug resistance in HIV infection.

SESSION VII: DESIGN AND DELIVERY OF VACCINES AND BIOPHARMACEUTICALS (continued)

Chairs: Irina Sominskaya, Ida Franiak-Pietryga

14.10 - 14.50 Karl Ljungberg (Karolinska Institutet, Stockholm, Sweden) DNA, RNA or viral particles: alphaviruses as vaccine vectors.

14.50 - 15.10 Irina Kholodnyuk (Riga Stradins University, Riga, Latvia) Nucleotide-modified RNA-aptamers selected to the cell-surface of Burkitt lymphoma cells suppress Burkitt lymphoma but not T-cell lymphoma cell proliferation in vitro.

15.10 - 15.50 Dzmitry Shcharbin & Maria Bryszewska (Lodz University, Lodz, Poland) Viral versus non-viral vectors for anticancer gene therapy.

IMMUNOTHERAPY OF MELANOMA

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The incidence of melanoma worldwide is increasing faster than any other type of cancer. The annual increase in incidence rate varies between populations, but in general lies in the range of 3-7% per year for fair-skinned Caucasian populations. Melanoma is almost always curable when it is found in its very early stages, however at the same time melanoma has the highest death rate of all types of cancer and is more likely to spread (metastasize) in the body. Until recently, the prognosis for patients with advanced melanoma has been very poor with a median overall survival (OS) of <1 year. Since 2011, seven new drugs have been approved by Federal Drug Administration (FDA) for the treatment of advanced melanoma, including three targeted therapies (for B-RAF mutated melanoma) and four immunotherapies with ipilimumab, pembrolizumab, nivolumab, and talimogene laherparepvec (T-VEC), and many more being in clinical development. The development of monoclonal antibodies (mAb) blocking specific inhibitory receptors expressed on tumor-specific T cells has significantly improved the clinical outcomes for patients with advanced melanoma. Neutralization of these molecules overcomes the exhaustion state of cytotoxic T lymphocytes (CTL) and restores their functional activities, which has led to durable complete and partial responses in a large number of patients. Ipilimumab targets the cytotoxic T lymphocyte-associated protein 4 (CTLA-4) receptor. Nivolumab and pembrolizumab target programmed cell death protein 1 (PD-1) receptors and have proven to be superior to ipilimumab alone, and the combination of ipilimumab and nivolumab has yielded higher response rates, greater tumor shrinkage, and longer progression-free survival than either monotherapy alone. Beyond their efficacy, these immunotherapies can be responsible for immune related adverse events, which need proper management. Moreover, the predictive response factors to these therapies remain elusive. T-VEC is an FDA approved oncolytic virus therapy for the treatment of stage III or IV melanoma that has relapsed after surgery. It is genetically modified live oncolytic herpes virus designed to replicate within cancer cells and produce an immunostimulatory protein - granulocyte-macrophage colony-stimulating factor (GM-CSF). The approval of T-VEC was based on the results of a large phase III trial that showed that 16% of patients treated with T-VEC had durable shrinkage of their tumors compared to 2% of those getting GM-CSF alone. Other therapies intended to ‘reeducate' T cells, such as tumor-infiltrating lymphocyte therapy, oncolytic viruses and tumor vaccines, have also yielded promising results and are under intense development. Taken together, current immunotherapy approaches for advanced melanoma fall into six main categories: checkpoint inhibitors, oncolytic virus therapies, cancer vaccines, adoptive T cell therapy, monoclonal antibodies, and cytokines. However, some patients with advanced melanoma still have a significant risk of mortality and there is the need for new successful therapies in patients with melanoma.

CANCER IMMUNOTHERAPY: ONCOLYTIC VIROTHERAPY IN LATVIA

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Background: Oncolytic virotherapy is a cancer treatment method using a virus that has the potential to halt the uncontrolled growth of a tumour or even destroy cancer cells. One central aspect in favour of oncolytic virotherapy is the selectivity for cancer cells *vs*. normal cells. The first oncolytic virus to be approved worldwide is a non-pathogenic ECHO-7 virus, Rigvir. It was registered in Latvia in 2004 for the treatment of skin melanoma.

Aim: The aim is to review recent clinical studies that evaluate the effectiveness of Rigvir in melanoma patients on time to progression and overall survival.

Results: Before approval and registration of the marketing authorization, Rigvir was tested in several clinical studies starting from 1968 that showed increased overall survival in melanoma, gastric and rectum cancer patients (1).

Since current clinical practice guidelines for stage I-II melanoma patients after surgical excision of tumour provide few, if any, treatment options, in two retrospective studies two patient groups were compared: Patients treated with Rigvir and patients observed according to guidelines.

The first retrospective study evaluated the time to progression of the disease in stage II melanoma patients (N=57). Recurrence of disease or metastasis was observed in 21 of 36 patients in the observation group, while only in 6 of 44 patients in the Rigvir group. The results show that the risk for disease progression is decreased by a factor of 6.67 by Rigvir treatment compared to observation (2).

The other retrospective study evaluated the overall survival in sub-stage IB, IIA, IIB, and IIC melanoma patients. Caucasian patients (N=79) were included in the study; 52 patients received Rigvir treatment, while 27 were observed according to guidelines. Cox analysis show that the melanoma patients treated with Rigvir had a 4.39-6.57-fold lower mortality than those under observation (3).

Discussion: The ECHO-7 virus Rigvir is first-in-class in oncolytic virotherapy and is approved for melanoma treatment. The recent retrospective studies show significant increase in time to disease progression and reduction in mortality in patients who are treated with Rigvir. Moreover, no record of any untoward negative side effects including increased toxicity grades has been reported.

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NK-MEDIATED CYTOLYSIS IN GLIOBLASTOMA, TRIGGERED BY VIRAL THERAPY

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Background. Based on immune phenotypes determined in GBM IV patients shortly before surgical intervention, increased absolute amounts of NK cells and TCRα/ß/CD8+ effector cells correlate with better survival.

Aims. The current study aimed at the characterization of GBM tumors as well as cell lines derived thereof. In addition, we questioned whether NK effectors activated by exogenous IL-2 and IL-15 may control from these tumors by their cytolytic function.

Results. We found that cell lines derived from GBM tumors expressed HLA-class I antigens to individually variable densities. In addition, we found high expression levels of the minor transplantation antigen MICA as well as NK receptors Trail DR5, CD155, and NKp30-Ligand, whereas HLA-class II antigens were not expressed. When testing autologous and allogeneic effectors against GBM tumors and K562 as a control, caspase 3/67 activities were strongly upregulated and tumor cell lysis could be demonstrated by IncuCyteZOOM^{TM}-assisted video microscopy. None of the patients tested, expressed tumor specific apoptosis to levels equally high as effectors derived from patients undergoing dendritic cell therapy combined with vaccination of xenogenic viruses such as NDV. Remarkably, NDV-treated patients' effectors also expressed increased expression densities of CD16 and DNAM-1.

Discussion. The analysis of 50 patients with GBM IV demonstrated increased survival in a subpopulation of patients who presented with increased amounts of NK cells before surgery. When these patients were compared with GBM IV patients undergoing viral therapy, the resulting effector cells expressed higher CD16 densities and increased cytolytic function against allogeneic GBM tumor cell lines. These results imply a beneficial effect by vaccination with xenogeneic viruses.

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EFFECT OF RIGVIR ON VIABLE CANCER CELL COUNT *IN VITRO*

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Background: Oncolytic viruses demonstrate tropism for neoplastic cells, thus, they selectively infect and kill cancer cells, but do not affect healthy cells. The first oncolytic virus approved for clinical use is Rigvir that was registered in Latvia in 2004 for treatment of skin melanoma. Rigvir is a non-pathogenic ECHO-7 virus that has not been genetically modified. A recent study shows that the melanoma patients that had been treated with Rigvir had a 4.39-6.57-fold lower mortality than those under observation according to current guidelines (1).

Aim: The aim of present study is to test the oncolytic effect of Rigvir on the viability of cell lines originating from human melanoma and other cancers using an automated real-time cell imaging system.

Methods: Cell lines of human rhabdomyosarcoma (RD), gastric adenocarcinoma (AGS), and lung carcinoma (A549) were from the American Type Culture Collection (ATTC) and metastatic cutaneous melanoma (FM-9) from the European Collection of Authenticated Cell Cultures (ECACC). Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% foetal bovine serum supplement and penicillin (100 U/ml) and streptomycin (100 µg/ml). Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air, and subcultured after trypsinization (0.25% trypsin/EDTA). Cells for tests were seeded on 6-well plates. When they reached approximately 10% confluency, Rigvir was added to the cell medium at final concentrations of 1% and 10% (v/v). An equal volume of DMEM medium (without Rigvir) was added to the control cells.

Results: Rigvir (10%) reduced by 90-100% the viable cell count of all tested cell lines (RD, AGS, A549 and FM-9) in a time- and dose-dependent manner.

Discussion: The present results suggest that all cancer cell lines tested *in vitro* independent of cancer type are sensitive to Rigvir.

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NANOPARTICLES AS THE BASIS FOR TREATMENT OF HIV/AIDS AND CANCER

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Background Short oligodeoxynucleotides (ODNs) and small interfering RNA (siRNA) are powerful tools for the gene therapy of intractable disease such as cancer and virus infections. However, the efficient and safe gene delivery systems are required to achieve the desired effects. A wide range of non-viral gene delivery carriers including cationic synthetic polymers, lipids and peptides have been developed and studied as alternatives to viral vectors. Among synthetic polymers, dendrimers and dendritic molecules have been proposed. Dendrons can functionalize materials such as poly(D, L-lactide-co-glycolide) acid (PLGA) nanoparticles, mesoporous silica or gold/silver nanoparticles. Their unique molecular architecture and properties make these nanoscaled materials highly interesting for the development of nanomedicine.

Aims The aim of the study was to examine different kinds of dendrimers and dendronized gold nanoparticles (AuNP) as potential delivery systems for ODNs and siRNAs in anti-HIV and anti-cancer therapy *in vitro.*

Results For this purpose we have checked the ability of nanoparticles to form complexes with ODNs and siRNAs using fluorescence and gel-electrophoresis. In the next steps we have characterized complexes formed in different molar/charge ratios using some biophysical methods: Z-potential (Laser Doppler Electrophoresis), hydrodynamic diameter (Dynamic Light Scattering), morphology and size (Transmission Electron Microscopy). Changes in the fluorescence polarization of labeled nucleic acids allowed us to examine time-dependent stability of complexes and their interactions with bovine serum albumin. We have tested nanoparticles for cytotoxicity, immunogenicity, nuclease resistance. Next, utilizing flow cytometry and confocal microscopy, we have checked transfection efficiency of naked nucleic acids and in complexes with nanoparticles.

Discussion There are better and worse candidates as non-viral vectors for gene delivery depending on the type of nanoparticles. Carbosilane and viologen-phosphorous dendrimers are good candidates for anti-HIV gene therapy, AuNPs capped with cationic carbosilane dendrons have anticancer activity.

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IMMUNOTHERAPY FOR HPV-RELATED DISEASE IN HIV-POSITIVE AND HIV-NEGATIVE MEN AND WOMEN

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Background HPV-related cancers are among the most common virus-associated cancers in HIV-negative men and women. HPV-associated anogenital cancer is one of the most common cancers among HIV-positive men and women, and the incidence of these cancers has been increasing as men and women live longer, despite improvements in anti-retroviral therapy. Immunotherapy for HPV-related disease has been challenging with a limited number of viral antigen targets. Several therapeutic vaccines have been tried with modest success, largely against pre-cancerous lesions rather than invasive cancers. None have yet been reported in the setting of HIV infection where immune dysregulation would be expected to lead to even greater challenges to achieving a satisfactory therapeutic result.

Aims 1) To summarize the pathogenesis of HPV-related disease and describe HPV protein expression in anogenital epithelium; 2) To describe recent studies of therapeutic vaccines against HPV-related disease and summarize the lessons learned about immune response to HPV from these studies; 3) describe HPV-HIV interactions in the epithelium and how these might influence the outcome of immunotherapy; and 4) and describe newer approaches to combining therapeutic vaccines with checkpoint inhibitors to potentiate vaccine efficacy

Results HPV-related lesions progress from initial infection of anogenital epithelium through development of high-grade squamous intraepithelial lesions (HSIL) and ultimately invasive cancer. The primary HPV genes expressed at all stages of cancer development are E6 and E7, and these are the targets of most HPV immunotherapeutic approaches. Th1-mediated immune responses, including CD8 cytotoxic T cell-mediated killing are likely important components of the immune response controlling HPV-related disease, with regression mediated by influx of tumor infiltrating lymphocytes. Treg cells also play a role and may be particularly important in attenuating immune responses in the setting of HIV infection. Other HIV-HPV interactions at the level of the epithelium may also play a role including modulation of tight junction integrity by the HIV-1 gp120 and tat proteins, and by host inflammatory cell proteins overexpressed in the setting of HIV-infected epithelium, including interferons and TNF-alpha (1). Newer approaches to HPV vaccination using electroporation to express HPV 16 and HPV 18 E6 and E7 have shown promise (2). A live-attenuated Listeria monocytogenes vaccine expressing HPV 16 E7 is also undergoing clinical trials.

Discussion PD-L1 is expressed on the surface of many tumor cells, virus-infected cells and antigen-presenting cells. Binding of PD-L1 to PD-1 on CTL cells leads to the inhibition of CTL proliferation and cytokine secretion, resulting in T-cell exhaustion and attenuation of immune responses. Inhibition of binding of PD-L1 to PD-1 releases the immune checkpoint blockade and may increase antitumor activity for some cancers. These drugs may be useful in combination with standard chemotherapy ad/or radiation therapy, particularly in the setting of immunocompromise such as HIV-1 infection, or in combination with therapeutic vaccines (3).

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BREAKING THROUGH LOW IMMUNOGENICITY OF REVERSE TRANSCRIPTASE IN THERAPEUTIC VACCINES AGAINST DRUG RESISTANCE IN HIV INFECTION

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Background: Recent vaccine trials demonstrated a potential of therapeutic HIV vaccines to reduce viral load in patients assisting antiretroviral therapy (ART). HIV immunotherapy becomes even more actual in view of the latest findings of an effective broad T-cell response clearing HIV-1 from the latent reservoirs. We hypothesized that a strong immune response against viral antigens responsible for drug resistance would create a bottle-neck to viral evolution forbidding or hindering the development of drug resistance that limits the efficacy of ART. Applied as a complement to antiretroviral treatment, anti-drug resistance immunotherapy would prolong the efficacy of ART.

Aims: To prevent or hinder the development of drug resistance in HIV/AIDS by DNA-immunizing against primary mutations of drug-resistance.

Results: Codon optimized genes of HXB2 HIV-1 clade B reverse transcriptase (RT) and RT with mutations of drug resistance were synthesized and clones into eukaryotic vector pVax1. Transfection with optimized RT genes resulted in ten-fold increase in expression of RT variants in cell culture. Immunogenicity of RT gene variants was accessed in DNA-immunized BALB/c mice. DNA immunized mice. RT-encoding plasmids were injected with needles or microneedles and electroporation (EP) of the injection sites was performed immediately after to increase the in vivo transfection efficacy. Optimal EP regimens were defined providing strong expression as well as efficient immune response. We have further tested if immunogenicity can be enhanced by repeating plasmid injection shortly after the prime (second prime). The latter enhanced immunogenicity, specifically the Th1-immune response. Boosting 3-4 weeks post primary injection gave further enhancement of immunogenicity in terms of cellular and antibody response against HIV RT. We have designed an “antigen challenge” model which allowed us to test the capacity of the immune response to clear HIV, here RT- expressing cells *in vivo*. For this, mice were primed with HIV genes, and boosted with HIV-1 genes mixed with luciferase reporter gene and reporter expression was monitored in days 1-23 by *in vivo* bioluminescence imaging. Model demonstrated the capacity of anti-RT response induced by primary immunization to efficiently clear RT/reporter co-expressing cells which was translated into a rapid loss of bioluminescence.

Discussion: Potency of HIV RT as a DNA immunogen can be greatly increased by codon-optimization of gene, optimization of delivery by electroporation and application of double prime, and prime-boost regimens.

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DNA, RNA OR VIRAL PARTICLES: ALPHAVIRUSES AS VACCINE VECTORS

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Background Viral vectors based on alphaviruses such as Semliki Forest Virus (SFV) elicit strong T cell and antibody responses with the ability to generate protective immunity in various disease models. In contrast to certain other viral vectors, alphavirus seroprevalence is generally low in most populations, and pre-existing immunity to alphavirus vectors has been shown to have little effect on induction of immune responses in animal models.

Aims On our vaccine platform, we aim to develop new and improved vaccines against a broad spectrum of infectious and malignant diseases.

Results One reason for the unparalleled immunogenicity of replication-deficient alphavirus replicons (VREP) may be the strong induction of innate immunity through interaction with various pattern recognition receptors such as PKR, RIG-I, MDA-5 and TLR 3 resulting in release of large amounts of type I interferon (IFN). Another important feature of VREP is that it promotes recognition of multiple, subdominant epitopes. Moreover, VREP immunization induces polyfunctional CD8+ T cell responses in terms of production of multiple cytokines (IL-2, IFN-γ and TNF) with a high and functional recall capacity. Importantly, alphavirus vectors can induce protective immunity in various disease models, both by intradermal, intramuscular and intranasal delivery. Interestingly, some alphavirus vectors promote mucosal homing of CD8+ T cells through up-regulation of the mucosal homing receptor α4β7 integrin.

Discussion There are considerable scale-up and safety issues that need to be adequately addressed for large-scale clinical development of recombinant alphavirus viral replicon vectors. To improve the safety as well as to address the production concerns, alphavirus replicon DNA and RNA vectors have been employed. This lecture will address alphavirus biology as well as their use as vaccine vectors as viral replicon particles, DNA-launched replicon vectors and RNA replicons. Further, we will discuss how vaccine modality, delivery and timing impact vaccine responses.

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NUCLEOTIDES-MODIFIED RNA-APTAMER SUPPRESSES BURKITT LYMPHOMA BUT NOT T-CELL LYMPHOMA CELL PROLIFERATION IN VITRO

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Background: Aptamers are oligonucleotides that can bind the ligands with high affinity and specificity by folding into complex tertiary structures (thought of as "chemical antibodies"). The SELEX (Systematic Evolution of Ligands by EXponential enrichment) technology for the generation of aptamers of the interest from the random sequences of combinatorial libraries was first described in 1990 by Tuerk&Gold[1] and by Ellington&Szostak[2]. Therapeutic and diagnostic applications of RNA-aptamers became possible since the nucleotides chemical modifications that protect from nucleases cleavage, had been introduced. Previously, we have elaborated the whole-cell SELEX method modification (Cell-SELEX-FA) for generation of nucleotides-modified RNA-aptamers (nmRNA-aptamers) to the cell-surface molecules[3].

Aims: To isolate nmRNA-aptamers that can distinguish between the Burkitt lymphoma (BL) cells and non-malignant lymphoblastoid (LCL) B-cells, and to investigate their potential application as blocking agents of BL cells proliferation.

Results: Using Cell-SELEX-FA, we have collected two nmRNA-aptamers pools that were selected to the cell surface molecules of the malignant BL Raji cells (1st pool) and non-malignant LCL cells (2nd pool). The nmRNA-aptamers pools were labeled with FITC and their specificity was shown by fluorescent laser-scanning microscopy. These nmRNA-aptamers were cloned; the individual clones were isolated, sequenced, and analyzed in order to separate unique and similar sequences. Two individual nmRNA-aptamers (Apt4 and Apt5) were examined for the cell-proliferation growth-blocking effect by direct cell counting and colorimetric MTT assay, using 4 BL, 2 T-cell lymphoma, and 1 LCL cell lines. Using cell counting test, the Apt5 growth-blocking effect for BL cell lines was observed after 48 and 72 hours at concentration 5.0 μM. After 72 hours, the number of alive Raji and Akata cells was reduced by 75% as compared to the controls. Both aptamers, after 72 hours of incubation, were inactive for the T-cell lymphoma cell line MT-2: the number of viable cells was only decreased by 10 and 15%. The MTT test revealed 42.3% inhibition of BL Raji cells proliferation for Apt5 and 48% inhibition of LCL cells proliferation for Apt4 at 72 hours after the aptamer input. Notable that for the T-cell lymphoma cell line Jurkat, inhibition of cells proliferation was 5.1% only for Apt5 in the same experimental setting.

Discussion: Generated nmRNA-aptamers, which can distinguish between malignant and non-malignant B-cells, can be implemented for the detection of malignant B-cells in the blood of patients with B-cell lymphoproliferative disorders. The new compounds (aptamer sequences) can be synthesized chemically and functional groups, such as fluorescence dyes or chemically reactive groups, can be readily attached to the nucleotides during the synthesis. The nmRNA-aptamers can be used as the carriers for drugs (small chemical molecules) and siRNA (for gene silencing) delivery inside the targeted cells. The nmRNA-aptamers selected for the B-cell lymphoma cells proliferation-blocking properties, should be further studied as the potential therapeutics *in vivo* by using experimental animal model.

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VIRAL VERSUS NON-VIRAL VECTORS FOR ANTICANCER GENE THERAPY

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Background. Gene therapy is one of the most effective ways to treat major infectious diseases, cancer, and genetic disorders. It is based on several viral and non-viral systems for nucleic acid delivery. The number of clinical trials based on application of viral and non-viral gene delivery systems is rapidly increasing.

Areas covered. This presentation discusses and summarizes recent advances in dendrimers as effective gene carriers *in vitro* and *in vivo*, and their advantages and disadvantages relative to viral vectors and other non-viral systems (liposomes, linear polymers) will be considered. In this regard, dendrimers are non-immunogenic and have the highest efficiency of transfection among other non-viral systems, and none of the drawbacks characteristic for viral systems. The toxicity of dendrimers both *in vitro* and *in vivo* is an important question that has been addressed on many occasions. Several non-toxic and efficient multifunctional dendrimer-based conjugates for gene delivery, along with modifications to improve transfection efficiency whilst decreasing cytotoxicity, are discussed. Twelve paradigms that impacted on the development of dendrimer-based gene delivery are described. The conclusion is that dendrimers are promising candidates for gene delivery, but it is early days and further studies are required before using them in human gene therapy.

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