Symposium

THE FOURTH SCIENTIFIC SYMPOSIUM: *APOPTOSIS AND NEOPLASMS*

Zagreb, March 28th, 2017

PREFACE

Scientific Symposium on Apoptosis and Neoplasms was held in Zagreb, Croatia, on March 28th '17 in the Library of Croatian Academy of Sciences and Arts, and organized by Department of Medical Sciences, Committee on genomics and proteomics in oncology.

Meeting started with two keynote lectures and ten brief overviews with various themes showing parts of the researches dealing with the study of the apoptotic mechanisms. There were 70 participants. One of the presented studies was from the Republic of Kosovo.

Symposium was dedicated to the Nobel Prize winners who signed up the appeal to stop aggression on Croatia.

The beginning of the Symposium was marked by two keynote lectures. Professor Nives Pećina Šlaus started by an overview presentation entitled: *So many „OMICS" and only one health*. She discussed the history of specialisations in molecular biology („omics") and the importance of multidisciplinary investigations in rapidly growing body of „molecular biology and medicine" research. During discussion, the participants emphasized the need for enhanced research coordination.

The second overview: *Metamorphosis, autophagocytosis, "whole body apoptosis" and neoplasms*, was presented by professor Mladen Belicza who spoke of oncogenesis being a part of evolutionary metamorphosis and apoptosis and proposed that investigations of human diseases pathogenesis should be integrated in health research of the entire biocenosis.

President of the Organizing Committee
Mladen Belicza

ABSTRACTS

of the 4th Scientific Symposium on *Apoptosis and Neoplasms*

ONLY ONE HEALTH, AND SO MANY OMICS

Nives Pećina-Šlaus

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The lecture was based on the article (N. Pećina-Šlaus and M. Pećina. Only one health and so many omics. Cancer Cell Int 15:64, 2015, DOI 10.1186/s12935-015-0212-2) in which the modern large-scale analyses have been reviewed. The title is paraphrasing the famous saying of Victor Schlichter from Buenos Aires children hospital in Argentina who said "How unfair! Only one health, and so many diseases". New approaches based on wide profiling methods in studying biological and medical systems are bringing large amounts of data typically stored in big data repositories. Several repositories important for human health today are: The Human Genome Project, The Cancer Genome Atlas (TCGA), The Cancer Genome Project, The Human Proteome Project (HPP), Human Epigenome Project, The Human Metabolome Database, Human Microbiome Project, The Human Connectome Project and The Human Exposome Project. They use specialized algorithms, analyze and present findings to be more comprehendable, thus introducing system sciences. The most common omics employed in the research of complex diseases are genomics -the analysis of complete genetic material of an organism –the complete nucleotide sequence of its DNA. The human genome is comprised of 3.2 billion nucleotides, but contains only 23,500 protein-coding genes. Closely connected to genomics are exomics, the part of the genome formed by exons. Exomes are the protein coding content of the genetic code and the human exome consists of 180,000 exons, roughly 1-2 % of our total genome. Transcriptomics study transcriptome that encompasses

all RNA molecules synthesized by the process of transcription, while proteomics can be defined as a large-scale study of proteins, their functions and structures. Since proteins are functional building blocks of cells, the information on proteome of a given cell or tissue in health or disease is a difficult but rewarding task to accomplish. Furthermore, we can also study epigenome (all epigenetic changes), metabolome (complete set of all metabolites in an organism), microbiome (all genomes of microbiota that symbiotically live in or on us), connectome (a map of all the neural connections of human brain) and exposome (the totality of exposures received by an individual during a lifetime). Today there is indeed a whole lot of omics and we wanted to stress the importance of future holistic approach in integrating the knowledge omics has rewarded us.

Keywords: omics; genomics; epigenomics; proteomics; metabolomics; microbiomics; exposomics; connectomics.

THE INTERPLAY BETWEEN MICRO-RNA MOLECULES AND APOPTOSIS-ASSOCIATED GENES IN HIGH-GRADE SEROUS OVARIAN CANCER

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Ovarian cancer is the seventh most frequent tumor type in women and the fifth leading cause of cancer-related deaths among women worldwide. In Croatia there are around 500 new cases annually while about 300 women die every year because of ovarian cancer. Its high death rate, particularly for a serous type which is the most frequent, is a result of the fact that most patients are diagnosed at an advanced stage of the disease. Therefore, there is a need for new knowledge about what causes ovarian cancer as well as new approaches toward better earlier diagnosis and therefore better effect on therapy.

One of the hallmarks of tumor cells is their ability to resist apoptosis, a process of programmed cell death. Deregulation of apoptosis plays a key role in the pathogenesis and progression of cancer, and leads also to chemotherapy resistance, what is a characteristic and one of the reasons of high mortality rate of high-grade serous ovarian cancer. According to the Kyoto Encyclopedia of Genes and Genomes

(KEGG) pathway database, at the moment there are 140 known apoptosis-associated genes in human genome. In addition, it is known that many microRNA molecules, a major class of small RNA molecules that post-transcriptionally inhibit gene expression, can regulate those apoptosis-associated genes. Therefore, there is a need for better elucidation of the interplay between microRNA molecules and apoptosis-associated genes.

In this talk I will present our research on microRNA and gene expression profiling of high-grade serous ovarian cancer. We used microarray technology which allows us to determine the expression patterns of more than 2,500 human microRNAs or over 26,000 protein-coding genes in a single experiment. Furthermore, from this sea of data, using various bioinformatic tools and databases, we were able to construct the microRNA:target gene interaction networks related to (dys)regulation of apoptosis in high-grade serous ovarian cancer. These results could help us to understand better the etiology of this type of ovarian cancer and to discover new diagnostic and prognostic biomarkers, as well as new targets for therapies.

Keywords: apoptosis genes; microRNAs; expression; microarrays; ovarian cancer.

THE ROLE OF DISHEVELLED GENE AND PROTEIN IN ASTROCYTIC BRAINE TUMORS

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Astrocytic brain tumors are the most common primary central nervous system neoplasms and are classified according to their lineage of origin, histology, behavior and prognosis into four WHO grades. Wnt signaling pathway is one of the basic mechanisms of cell signaling as evidenced by its participation in a numerous processes in the cell. The most interesting role of this signaling pathway for us is its involvement in brain tumorigenesis. We aim to investigate the incompletely understood role of Dishevelled (DVL) gene family, which is considered to be the central hub of wnt signaling.

Genetic changes of *DVL1*, *DVL2* and *DVL3* genes were analysed by PCR/loss of heterozygosity (LOH)/microsatellite instability (MSI) methods using Spreadex elec-

trophoresis. Protein expressions and localizations of DVL1, DVL2 and DVL3 proteins were analyzed by immunohistochemistry.

In this study, results showed that DVL expression is significantly higher in astrocytomas than in normal brain tissue. Furthermore, expression increases with the pathological grade of tumors.

DVLs may play a important role in formation and invasion of astrocytic brain tumors. Future studies using an expanded cohort may help to improve the understanding of the role of individual DVL in astrocytoma as well as its potential as a molecular diagnostic marker or a therapeutic target.

Keywords: DVL1; DVL2; DVL3; Wnt signaling pathway; astrocytic brain tumors.

EXPRESSION OF SURVIVIN IN INVASIVE AND NONINVASIVE UROTHELIAL CARCINOMA OF THE URINARY BLADDER

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Objective: The inhibitor of apoptosis protein survivin regulates apoptosis and cell cycle.There are conflicting data in the literature regarding relationship between the degree and localisation of survivin expression and behavior of urinary bladder carcinoma. Here, we correlated immunohistochemical localization of survivin with the histologic diagnosis of noninvasive and invasive urothelial carcinoma of the bladder (UCB).

Methods: A total of 82 histopathologically confirmed UCB were recruited. Of these 32 were non-invasive, 27 had invasion of lamina propria and 23 had confirmed invasion ofmuscularispropria of the urinary bladder. Immunohistochemistry was used to detect survivinexpression in tumor tissues. The intensity of the reactions was assessed semiquantitatively, using three expression categories; 0-5% (low expression), >5%-50% (moderate expression), and >50% (high expression).

Results: Higher survivin expression was found in less invasive UCB (p=-0,011 for nuclear and p<-0.001for cytoplasmic expression) and in UCB with lower histological grade (p=-0,018for nuclear and p<-0.001 for cytoplasmic expression).

Conclusions: Our results suggested that high survivin expression was associated with tumor stage and grade in UCB. Further studies are needed to conclude if survivin expression can be used as a diagnostic or prognostic marker for UCB.

***Keywords*:** surviving; urothelial carcinoma; urinary bladder; immunohistochemistry.

EPIGENETIC AGENTS INFLUENCE PROLIFERATION AND APOPTOSIS IN MOUSE TERATOCARCINOMA IN VITRO

Jure Krasić [1], Robert Buljubašić [2], Maja Buljubašić [3], Ana Katušić [1], Maja Vlahović [1], Florijana Bulić-Jakuš [1], Monika Ulamec [4,5], Nino Sinčić [1]

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Testicular Germ Cell Tumours (TGCT), although rare, are the most frequent malignancies in young male population and believed to be initiated by epimutations, i.e. aberrant epigenetics, already *in utero*. Among various, teratoma is the most differentiated TGCT type encompassing all three germ layer derived tissues. Mouse teratoma is a well-established *in vitro* model which may be obtained by cultivating 7,5–days-old C3H mouse embryos and represent an ideal system to investigate the effect of the most prominent epigenetic drugs and agents.

After embryo isolation, they were treated for two hours with 5-azacytidine, Trichostatin A, Valproat, esiNanog, esiOct3/4 and esiTrrap, respectively. Embryos/teratomas treated with esiGFP served as a negative control. The embryos/teratomas were measured on day 0 and for the consequent 7 days of culturing, after which teratomas were scrapped, Sainte-Marie fixed and paraffin embedded for IHC analyses.

Epigenetic drugs and agents reduced significantly teratoma growth, with the exception of esiNanog and esiTrrap. Most prominent decrease in growth was determined in 5-azaC and esiOct3/4 treated embryos/teratomas.

IHC analysis of proliferative activity showed significant rise in Ki-67 signal in esiNanog and esiTrrap, as well as in 5azaC treated embryos/teratomas, compared to control. Apoptotic activity showed no significant change in any treatment.

This preliminary data notifies that epigenetic drugs and agents may have a significant effect on embryo/teratoma growth. It seems that teratoma growth is inhibited by necrotic activity rather than apoptosis which could consequently induce a rise in proliferation as a tissue reaction.

Keywords: epigenetics; DNA methylation; apoptosis; proliferation; TGCTs; mouse teratocarcinoma in vivo.

APOPTOSIS IN NATIVE VEIN WALL IN FAILURE OF HEMODIALYSIS ARTERIOVENOUS FISTULAS

Laura Leci-Tahiri [1], Božo Krušlin [2] , Majda Vučić [2], Zdenko Sonicki [3], Floriana Bulić-Jakuš [4], Ivo Lovričević [5]

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In line with the growing interest for studying apoptosis in blood vessels, this study is one of the first to assess the apoptosis in native veins used for arteriovenous fistulas (AVF) done for hemodialysis. The aim of this study was to evaluate apoptosis in previously punctured native veins (study groupy) compared with not punctured native veins (controls) in patients who undergo a surgical procedure for AVF as dialysis access. Cephalic vein specimens were obtained from 60 patients before the placement of AVF. Half of the specimens were from the previously punctured and half from non-punctured veins. A 1-cm long segment was excised from distal part of the cephalic vein, divided into two portions along longitudinal axis and prepared for immunohistochemical analysis. Immunohistochemical assessment and quantification of signals was used to evaluate the expression of Bax, p53, caspase 3 and Bcl-2.

Vein specimens from the study group with previously punctured veins showed significantly increased caspase 3 and Bax expression, compared with the control

group (p<0.01). Bcl-2 expression in the study group was significantly decreased compared with the control group (p<0.01). p53 showed no significant differences between the two groups (p=0.791). There were statistically significant differences in fistula failure between the study group and control group (26.7% *versus* 6.7%, p=0.038). The association we have found between previously punctured veins and apoptosis indicates the role that venipuncture may play in the development of apoptosis. Patients with increased apoptosis showed an increased fistula failure, which is of importance for the improvement of the AVF procedure itself.

*Keywords***:** native vein wall; arteriovenous fistula; hemodialysis; apoptosis; fistula failure.

THE ASSOCIATION OF *BIRC5* GENE POLYMORPHISM AND SURVIVIN EXPRESSION IN DIFFERENT TUMORS TYPES

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Survivin, encoded by *BIRC5* gene, belongs to the family of inhibitors of apoptosis (IAP) proteins. Survivin is an essential chromosomal passenger protein required for mitotic progression. It is also an inhibitor of apoptosis and can prevent caspase-mediated cell death. It is usually expressed in embryonic tissues and homozygous survivin deletion results in early embryonic death, showing its essential role in cell development, differentiation and homeostasis. In healthy organisms it is not expressed in differentiated tissues, while its expression is markedly increased in most cancers (including bladder cancer, lung cancer, breast cancer, stomach, esophagus, liver, ovarian cancers and hematological cancers).

In tumors its presence correlates with increased resistance to chemo- and radiotherapy, as well as worse survival rate. Although its expression is usually confined to G2-phase and mitosis, survivin is in cancer often expressed throughout the cell cycle.

At least 5 different splice variants of the survivin gene have been reported in humans so far (wild type, 2α, 2B, 3B and deltaEx3). All survivin protein isoforms arising from the splice variants share the same N-terminus region, but differ in the carboxyl end. The transcript expression levels of various survivin isoforms have been significantly associated with clinico-pathologic characteristics in several cancers.

Several BIRC5 polymorphisms in promoter and 3'UTR regions were studied in various types of cancer, and were found to be correlated with susceptibility (gastric, bladder and hepatocellular), survival (colorectal and breast) or age of onset (ovarian cancer).

In this study we investigated the role of *BIRC5* polymorphisms and survivin gene expression in several types of cancer. 74 normal samples, 48 oral and oropharyngeal squamous cell carcinoma (SCC) samples, 35 breast cancer (BC) samples and 40 ovarian carcinoma (OC) samples were typed for BIRC5 polymorphisms using high resolution melting analysis and Sanger sequencing. For samples with available tumor tissues, either fresh frozen (48 SCC samples and 23 OC samples) or paraffin embedded (26 BC samples), survivin expression was measured with qPCR or immunohistochemistry. For OC samples, levels of different survivin isoforms were also determined.

19 polymorphisms were found, 7 were found in promoter region, 1 in 5'UTR, 4 in coding region and 7 in 3'UTR. 10 polymorphisms were found in all 4 groups, 5 in 3 groups, 1 in 2 groups and 3 in only 1 group of samples. Two of the polymorphisms that were found only in one sample each have not previously been reported. Both, one in exon 2B (c.221+1199G>A), and second, in 3'UTR (c.9349G>C) were found in SCC samples.

47 SCC samples showed survivin mRNA expression. 24 BC samples showed protein survivin expression. All 23 OC samples expressed wild type survivin, but isoform expression varied greatly. The highest expression of all splice variants was of survivin 2α, then wild type survivin, followed by survivin deltaEx3 and survivin 3B. The lowest expression was of the splice variant survivin 2B, which was expressed in only 16 of 23 samples analyzed. All splice variants had higher expression in ovarian cancer compared to healthy Fallopian tube tissue.

Keywords: surviving; BIRC 5; squamous cell carcinoma; ovarian cancer; breast cancer.

CHANGES IN APOPTOTIC ACTIVITY DRIVEN BY DNA METHYLATION EPIMUTATIONS IN HUMAN CANCER

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Apoptosis is one of the fundamental biological phenomena, well known to be crucial in cancer development for its vital role in a subtle interplay between proliferation and cell death. Programmed cell death is indeed a powerful physiological frontier cancer cells have to overcome along the way of fulfilling their full malignant potential. Therefore, complex signalling networks developed in the frame of apoptosis which can be crashed not only by gene mutations but disruption in gene expression regulation as well. DNA methylation is one of the most prominent epigenetic mechanisms strongly effecting gene expression by organizing chromatin status and its permissiveness to transcription. Aberrant DNA methylation is an epimutation already recognized as an early event, sometimes even preceding other well-known molecular carcinogenic incidences. Reports from literature, which will be discussed in this lecture, point to DNA methylation epimutations as underlying mechanism of aberrant apoptotic gene expression resulting in a breach of cell homeostasis regulatory mechanisms toward carcinogenesis.

Keywords: Epigenetics; DNA methylation; apoptosis; epimutation; cancer.

APOPTOSIS AND COLORECTAL CANCER

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Programmed cell death, or apoptosis, is a fundamental mechanism of tissue homeostasis. In the normal colon, as much as 10^{10} cells per day undergo apoptosis and are shed into the lumen. One of the hallmarks of cancer is escape from mechanisms regulating apoptosis, such as cellular response to extrinsic growth factors

and telomere shortening. Escape from apoptosis also makes cancer cells resistant to chemotherapy and radiation. The molecular machinery of apoptosis is a potential target for drugs that could promote cell death in colorectal cancer cells. The intrinsic (mitochondrial) pathway of apoptosis is regulated mainly the the Bcl-2 protein family, which are activated by various stimuli, including growth factor withdrawal and cellular damage. Under such circumstances, the antiapoptotic factors (eg. Bcl-2, Bcl-XL, Bcl-W, Mcl-1) release the proapoptotic factors (eg. BAK, BAX), which in turn increase mitochondrial membrane permeability, leading to cytochrome C release and Caspase 9 activation and consequently, via Caspase 3 activation, to apoptosis. In cancer antiapoptotic Bcl-2 family members are overexpressed, while the proapoptotic factors are underexpressed. "BH-3 mimetics" are a class of small molecules inhibitors of certain antiapoptotic Bcl-2 family members. Navitoclax is a potent inhibitor of Bcl-2, Bcl-W and Bcl-XL, with in vitro evidence of apoptosis induction in colorectal cancer cells, and demonstrated safety in a phase I clinical trial in combination with established therapy regimes. Obatoclax is a promising pan-Bcl-2 inhibitor. HA14-1 is a small molecule selectively targeting Bcl-2. Oblimersen is an antisense oligonucleotide targeting Bcl-2, and has shown safety in phase I trials. In summary, several new drugs focusing on apoptosis induction in colorectal cancer are currently being studied, which will hopefully lead to better therapies in the future.

Keywords: apoptosis; colorectal cancer; therapy; new drugs.

KIDNEY TUMOR EMPERIPOLESIS

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Emperipolesis means active penetration of one cell by another which remains intact. Main cells participating in emperipolesis usually are histiocytes, but other cell types may participate. Emperipolesis can also be found in some cancers.

The exact mechanism for emperipolesis is not fully known but it is believed that the pathway of lizosom degradation mediated NK ("natural killer") cells is crucial in this process. It was noted that NK cells use emperipolesis when leading tumor cells to programmed cell death. Process similar to emperipolesis, called entosis was also described, but with the different ultrastructural pathways and molecules in-

volved. Fate of the cells after process is done is one of the raised questions. Usually degradation of one cell is seen.

In some epithelial tumors of the kidney emperipoplesis was observed. In the last 5 years an entity called biphasic squamoid-alveolar carcinoma of the kidney was described and it has characteristics of papillary renal cell carcinoma with emperipolesis in the near proximity of large cells. The significance of this process in the kidney tumors has not yet been fully clarified.

Keywords: emperipolesis; kidney tumor; biphasic squamoid-alveolar carcinoma.

COMPARISON BETWEEN SURVIVIN AND KI-67 PROLIFERATIVE INDEX IN HER-2 POSITIVE AND TRIPLE NEGATIVE BREAST CARCINOMA

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Survivin is a member of the inhibitor of apoptosis (IAP) family. It is also involved in the regulation of cell division. Survivin is widely expressed in foetal tissues and in human cancers, but generally not in normal adult tissue. Apoptosis is the process of programmed cell death where senescent or damaged cells that are beyond repair are eliminated. It is a cascade of molecular events regulated by proteins that promote or prevent cell death. It is believed to be an important mechanism by which therapeutic chemotherapy and radiation therapy destroy cancer cells. Survivin is an anti-apoptotic protein that is overexpressed in most human cancers. Survivin regulates the G2/M phase of the cell cycle by associating with mitotic spindle microtubules, and it directly inhibits caspase-3 and caspase-7 activity. During tumorigenesis, survivin expression is inversely correlated with apoptosis inhibition and positively correlated with proliferation and angiogenesis. In our previous research, we observed immunohistochemical results of survivin and the relation between survivin and proliferative index Ki-67 in 50 cases of breast cancer in accordance with immunophenotype by St.Galen (2015g.). Survivin immunoreactivity was evaluated semiquantitatively according to the previous studies. Nuclear and cytoplasmic tumour cell immunoreactivities were separately assessed at 40 magnification, and were given an arbitrary score as follows: 0(0-5% positive cells); 1(5–20%); 2(21–50%);

3(51–75%); 4(>76%). A cutoff value of 20% was established as a positive result. Immunohistochemical analysis showed positive expression for survivin in 18 of 50 cases (36%) of breast carcinomas of TNM stages I to III. What we should emphasize in our study is the correlation between Her 2-positive tumours and survivin expression (p=0,007). What we can interpretate in our research is, that survivin is not expressed in luminal A or luminal B types of tumours, and its expression in nucleus is an indicator of better prognosis which is consistent with previous studies. Strong association of survivin expression in cytoplasm in Her-2 positive tumours is a predictor of unfavourable outcome. What stays unclear in our study is lack of survivin expression in triple negative tumour types, or positivity in small sized tumours which does not comply with results in previous studies. Our goal is to make larger study that would comprise 30 cases of HER-2 positive and 30 cases of triple negative tumors. We want to compare survivin immunoreactivity and proliferative index Ki-67 with other prognostic parameters such as tumor size, age of the patients, tumor grade and oestrogen receptor immunoreactivity.

Keywords: apoptosis; survivin; breast cancer.