Symposium

THE FIFTH SCIENTIFIC SYMPOSIUM: *APOPTOSIS AND NEOPLASMS*

PREFACE

The *5th Symposium on Apoptosis and Neoplasms* was held in Zagreb, Croatia, on March 27th 2018 in the Library of Croatian Academy of Sciences and Arts, organised by Department of Medical Sciences, Committee on genomics and proteomics in oncology. There were 10 presentations and 80 participants.

In an attempt to promote translational medicine, the goal of the *5th Symposium on Apoptosis and Neoplasms* is to develop new research approaches into the complexity of biological systems and their regulation, especially in the field of apoptosis and oncogenesis. Thus, this symposium helps in reducing various barriers in order to establish better collaboration between all scientists and researchers, and improves the development of multidisciplinary research. In this sense, we hope to contribute to the redevelopment of translational and clinical science. Eight open topics followed after two introductory lectures, *Evolutio-Conditio sine qua non*, and *Macroevolutionary aspects of oncogenesis*. These two introductory lectures presented recent data in research and interpretations of the theory of evolution and oncogenesis and have been conected to the overview: *Metamorphosis, autophagocytosis, "whole body apoptosis" and neoplasms.* This overview was presented at the *4th Symposium on Apoptosis and Neoplasms*, organised in 2017. by professor Mladen Belicza, who spoke on oncogenesis being a part of evolutionary metamorphosis and apoptosis, and proposed that investigations into the pathogenesis of human diseases be integrated in health research of the entire biocenosis.

The eight open topics, *The role of postreplicative mismatchrepair* (*MMR*) *in tumorigenesis, Liquid biopsy in oncology – current achievements and new challenges, Use of free DNA in tumor diagnostics, Comparative analysis of apoptotic activity in testicular tumors and mouse teratocarcinoma animal model, Confocal laser scanning microscopy at the Ruđer bošković Institute, p53/p63/p73 protein network in human tumors, Cell block - view below morphology level, Valproate enhances apoptosis of gastrulating mammalian embryo cultivated in vitro,* represented an actual part of croatian investigation in multidisciplinary cancer research.

President of the Organising Committee of the Symposium
Mladen Belicza

ABSTRACTS
of the 5th Symposium on Apoptosis and Neoplasms

EVOLUTION – CONDITIO SINE QUA NON

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It is a known fact that nothing in biology make sense except in the light of evolution. Hence, we can stretch that famous saying by the late and great Theodosius Dobzhansky to saying that nothing in modern medicine makes sense except in the light of biology.

One of the greatest scientific theories, some will dare to say the greatest scientific theory our species ever came upon - The Theory of Evolution is interestingly enough one of the most unaccepted, if not the most unaccepted scientific theory yet to this day (*Figure 1*).

There are big questions in life and science and then there are crucial ones! What is Universe? What is life? Where did it all begin? How big it is? Where did we come from? In a brief overview the aim is to try to explain amazing facts about Life and the Universe we live in that can only scientifically be explained by the Evolution. From first few seconds to flight to the Moon…from first cells to ecosystems…from first societies to global networks. Greatest voyage ever through biological thinking and its importance and implementation in modern medicine. One brief overview from first stars to our species in one unbroken chain of time and space glued together by one phenomenon ... the Evolution.

Keywords: theory of evolution; origin of life; human evolution.

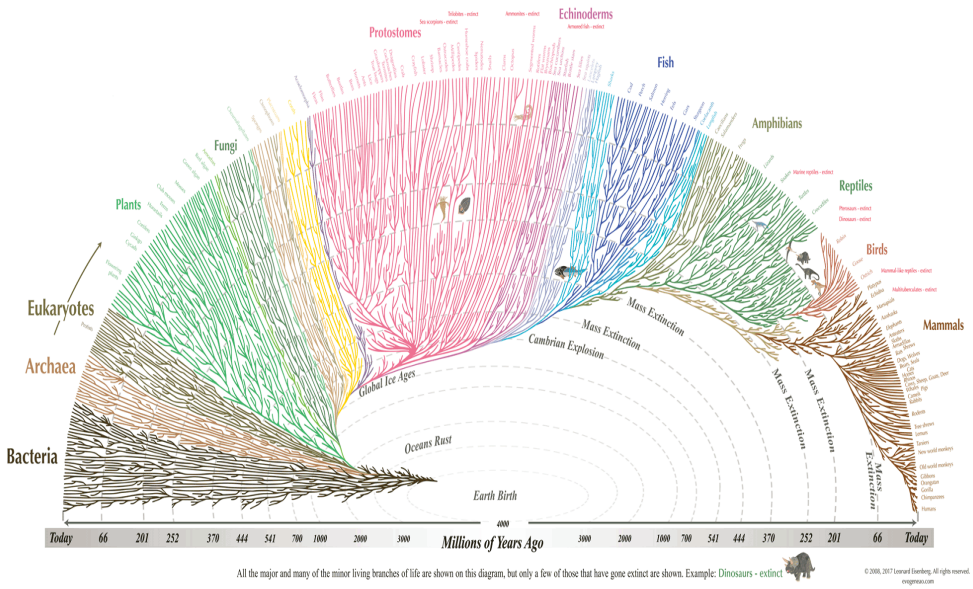

Figure 1. Tree of life with all the major and many minor branches of life with few extinct branches shown, for example Dinosaurs (Leonard Eisenberg, 2017; from www.evogeneao.com).

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THE ROLE OF POSTREPLICATIVE MISMATCHREPAIR (MMR)IN TUMORIGENESIS

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Impaired cellular DNA mismatchrepair (MMR) mechanisms are involved in cancer initiation and progression. Cancer cells are thought to have a mutator phenotype in which an elevated rate of mutation persists. The defective functioning of MMR proteins will consequently give rise to microsatellite instability (MSI). The importance of MSI phenotype in human tumors is an emerging field demonstrating its involvement in the clinical course of the disease, response to therapy, and survival outcomes (Clark et al 2013, Hause et al, 2016). The MMR system, which consists of a group of proteins specialized in recognizing mispaired bases and small loops of insertion or deletion, checks the post-replicated DNA strand. MMR machinery is coded by 8 genes in the human: hMLH1 (3p21), hPMS1 (hMLH2) (2q31.1), hMLH3 (14q24.3), hPMS2 (hMLH4) (7q22.2), hMSH2 (2p21), hMSH3 (5q14.1), hMSH5 (6p21.3), hMSH6 (2p16) (Fishel 2015; Loeb, 2016; Modrich 2016). Variations and alterations in DNA repair genes are important factors for specific tumor susceptibility.

In a group of 50 intracranial meningiomas, we investigated the involvement of two major MMR genes, MLH1 and MSH2, using microsatellite markers D1S1611 and BAT26 amplified by polymerase chain reaction and visualized by gel electrophoresis on high-resolution gels. Furthermore, genes DVL3 (D3S1262), AXIN1 (D16S3399), and CDH1 (D16S752) were also investigated for microsatellite instability (Pećina-Šlaus et al, 2017a). Our study revealed constant presence of microsatellite instability in meningioma patients when compared to their autologous blood DNA (Pećina-Šlaus et al, 2016a). Altogether 38% of meningiomas showed microsatellite instability at one microsatellite locus, 16% on two, and 13.3% on three loci. The percent of detected microsatellite instability for MSH2 gene was 14%, and for MLH1, it was 26%, for DVL3 22.9%, for AXIN1 17.8%, and for CDH1 8.3%. Since markers also allowed for the detection of loss of heterozygosity, gross deletions of MLH1 gene were found in 24% of meningiomas. Genetic changes between MLH1 and MSH2 were significantly positively

correlated (p=0.032). Wealsonoted a positivecorrelationbetweengeneticchanges of MSH2 and DVL3 genes (p=0.034). No significant associations were observed when MLH1 or MSH2 was tested against specific histopathological meningioma subtype or World Health Organization grade. However, genetic changes in DVL3 were strongly associated with anaplastic histology of meningioma ($\chi 2$ =9.14; p=0.01).

We believe that our study contributes to a better understanding of the genetic profile of human intracranial meningiomas and suggests that meningiomas harbor defective cellular DNA MMR mechanisms (Pećina-Šlaus et al, 2016b; Pećina-Šlaus et al, 2017b). Loss of proper functioning of DNA damage repair genes and proteins, whether through mutations or loss of their expression, is strongly correlated to the introduction of genomic instability and consequent tumorigenesis.

Keywords: meningioma; microsatellite instability; loss of heterozygosity; MLH1 MSH2; BAT26; D3S1611; AXIN1; CDH1; DVL3.

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LIQUID BIOPSY IN ONCOLOGY – CURRENT ACHIEVEMENTS AND NEW CHALLENGES

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In the era of precision oncology, decisions about treatment of cancer patients are often being based on genetic and other specific characteristics of a particular cancer in a particular patient. To analyze these characteristics we need to obtain cancer tissue sample through tissue biopsy, which is often an invasive, risky and painful procedure. Furthermore, due to cancer clonal evolution and heterogeneity, biopsy samples from one lesion (primary cancer or metastasis) might not be relevant and representative for other cancer lesions in the same patient. A potential solution for these problems is liquid biopsy, where cancer characteristics are determined by analysis of bodily fluids, most often blood.It was shown that circulating tumor cells (CTC), circulating cell-free tumor DNA (ctDNA) and circulating cancer exosomes can be detected and characterized in blood samples from cancer patients.Many recently published studies have shown potential clinical utility of liquid biopsy for early cancer detection, making decisions about treatment, monitoring treatment response, and predicting patient prognosis in different types of cancer. However, there are some technical, clinical, and biological challenges that need to be addressed before liquid biopsy tests become routine standardized procedures for cancer patients. So far, onectDNA based liquid biopsy test was approved by U.S. Food and Drug Administration in 2016 for detecting *EGFR* mutations in patients with lung cancer.

Keywords: liquid biopsy; cancer; circulating tumor cells; exosomes; cell-free tumor DNA.

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ON THE TRACK OF CELL-FREE DNA TECHNOLOGY

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Cell free DNA (cfDNA) technology is a crucial segment in liquid biopsy concept. Its implementation in clinical practice would represent a significant improvement in cancer patient management. It is usually less invasive, subject to less post-procedure risks and complications, less painful and therefore more acceptable to patients. CfDNA sampling usually has lower infrastructural demand and can be performed in a less equipped medical facility by less specialized personnel. Still, after-sampling procedures mostly involve state of the art molecular biology analyses on edge technology carried out by highly specialized medical laboratory personnel (1,2).

Although cfDNA is widely accepted as a term describing DNA in blood serum or plasma, it represents just a DNA fragment outside a cell. Therefore, cfDNA is present in liquid tissues, like blood indeed, but in any body fluid as well. In fact, cfDNA should be present in any non-cellular biological sample that was in contact with a cell (3).

CfDNA is a pool of fragmented cellular genomic DNA physiologically released in extracellular spaces, mostly body fluids and blood during the process of apoptosis. Therefore, the human body is constantly flooded with low and variable amounts of cfDNA. Furthermore, cfDNA can be actively secreted outside a cell as well, but then we refer to it as cfDNA circulating enclosed in exosomes. Expected concentration of cfDNA can be further increased in conditions of higher physiological demand, e.g. physical activity or pregnancy (1).

Higher concentration of cfDNA is expected in pathological conditions like inflammation, immune diseases, trauma, insults etc. Indeed, any condition with stronger cell death activity, especially necrosis, raises the amount of cfDNA. Highest concentration of cfDNA was mostly reported in patients with cancer. Fraction of cfDNA originating from cancer cell gDNA is referred to as circulating tumour DNA (ctDNA).

Low amount of cfDNA in a sample, often combined with minute sample size, makes methods of high yield DNA isolation a necessity. Many have already been developed and commercialized. DNA isolation methods involving silica columns or magnetic bides seems to meet the challenge but no consensus which should be standardly used has yet been achieved. Namely, concentration of isolated cfDNA range from less than 1 to more than 100 per 1 mL of sample.

As mentioned above, cfDNA represent fragmented gDNA released in extracellular spaces mostly in a process of cell death. Therefore, every genomic or epigenetic incident in gDNA should be found in cfDNA. This finding is the very stronghold of all the attempts of translating cfDNA technology in clinical practice, especially cancer management. Still, it has to be kept in mind that altered cfDNA is just a fraction of the total circulating cfDNA corresponding to pertaining population of (epi)mutated cells in a body of an individual. This furthermore highlights the necessity of outstanding power of DNA isolation methods needed. Indeed, contribution of altered DNA, e.g. ctDNA, can vary from less than 0.01% to 60% in overall isolated cfDNA (3).

Congruently, molecular biology methods by which cfDNA should be analyzed has to have high sensitivity, but most of all an outstanding power when using extremely small intake of DNA. Next generation sequencings and digital droplet PCR are believed to cope with this challenge, and are wildly recommended. Still, less powerful technology, like Sanger sequencing or real-time PCR, can successfully address less demanding tasks (3,4). Never the less, all methods should be performed by highly educated personnel in specialized

facilities keeping in mind all technological limitations that have yet to be addressed.

Keywords: cell-free DNA; circulating tumour DNA; liquid biopsy.

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COMPARATIVE ANALYSIS OF APOPTOTIC ACTIVITY IN TERATOCARCINOMA AND THE EXPERIMENTAL MOUSE TERATOCARCINOMA MODEL

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Incidence of testicular germ cell tumors (TGCT) has been continuously rising (1-5% per year), (1). In Croatia the incidence of TGCT is increasing at the highest rate in the world. Croatia also has a high rate of mortality compared to other countries (2).

Germ cell neoplasia in situ (GCNIS) is a precursor lesion of TGCT's which makes up to 95% of all testicular tumors. TGCT are divided into pure seminomatous tumors and mixed germ cell tumors (non-seminomas).

GCNIS is considered to be driven by an interplay of genetic, epigenetic and micro-environmental factors that lead up to an arrest of gonocyte differentiation (3,4). GCNIS is reprogrammed in the development of non-seminomas into embryonal carcinoma (EC) cells, which make up the pluripotent core of the non-seminomas (3). EC cells are highly similar to embryonic stem cells, expressing the same pluripotency markers OCT4, SOX2 and NANOG. EC cells differentiate into teratoma tissue, or retain their pluripotency and high malignancy as in teratocarcinoma (5).

The well-known and established experimental mouse model for teratocarcinoma, at the Biology Department of Zagreb's School of Medicine, has been described based on histological/histochemical methods and its molecular signature has not yet been studied (6). The aim of this study was to compare the rate of apoptotic activity in the experimental mouse model as well as in human teratocarcinomas.

Caspase-3 is the most important apoptotic executioner caspase in apoptosis and is activated by both intrinsic and extrinsic pathways. Some authors consider that Caspase-3 plays a critical role in ES cell differentiation by negatively regulating the self-renewal machinery of these stem cells, in part by cleaving Nanog (7,8).

Formalin-fixed paraffin-embedded tissue from 10 testicular teratocarcinoma from the Ljudevit Jurak Pathology and Cytology Department Archive and 10 animal model tumors from the Biology Department of Zagreb's School of Medicine were used for immunohistochemical detection of Caspase-3. Slides were analyzed semi-quantitatively, at the area of strongest reaction, by two pathologist (S.M. and M.U.), on a scale from 0-3, depending on the percentage of reactive cells. The data were analyzed in GraphPad Prism using the Mann-Whitney test.

The results have shown a statistically significant difference in the rate of apoptosis between the human teratocarcinomas and the experimental mouse model, with the mouse model showing a higher rate (more than 25% of positive cells) in 64% of tumors compared to 30% of human tumors with highest reaction.

The difference could be in part attributed to the fact that the study was a pilot with a relatively small sample pool, and the difference in biological development and "age" between the human and mouse model teratocarcinomas which was obtained uniformly. Western Blot analysis of Caspase-3 activity should be done to verify the results and a bigger cohort should be studied (*Figure 1*).

Keywords: TGCT; mouse model; apoptosis; Caspase-3.

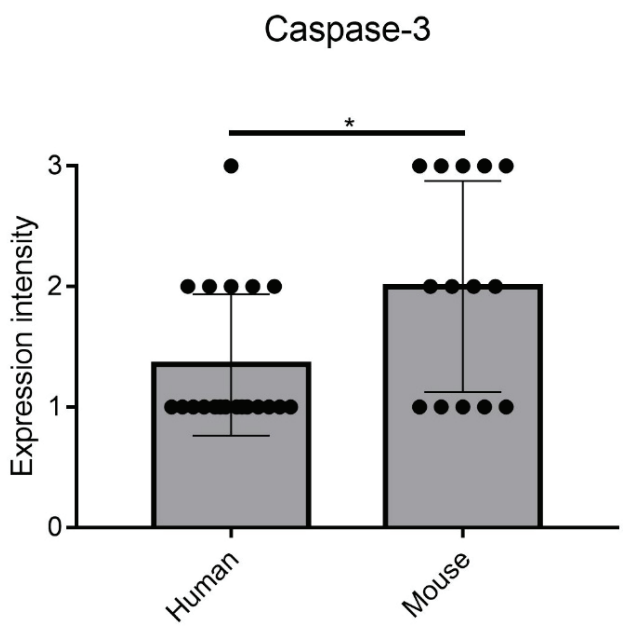

Figure 1. Caspase-3 expression intensity in teratocarcinoma and mouse experimental teratocarcinoma model.

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CONFOCAL LASER SCANNING MICROSCOPY AT THE RUĐER BOŠKOVIĆ INSTITUTE

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Fluorescence microscopy has been extensively used over more than a century to study the localization and dynamics of fluorescently labeled macromolecules in fixed and living cells and tissues [1]. In the last 20 years, confocal microscopy has become the dominant form of light microscopy used in biomedical research, including oncology [2]. The main advantage of the confocal fluorescence microscopy, in comparison to wide-field fluorescence microscopy, is its capability to

suppress the out-of-focus light emanating from the specimen, which results in crisp images of a focal plane thinner than one micrometer. Confocal microscope can thereby perform optical sectioning resulting in a three-dimensional map of the fluorophore distribution within a fluorescently labeled specimen.

Centre for confocal microscopy at the Ruđer Bošković Institute currently operates two laser scanning confocal microscopes (*Table 1*). The SP8 instrument can simultaneously use 8 arbitrary excitation lines within the visible spectrum and 4 detectors with freely adjustable detection ranges. These features open up the capability to obtain systematic characterization of a specimen's two-dimensional excitation/emission profile and thereby to perform microspectrofluorimetry *in situ*. Altogether, 6 fundamental imaging parameters are configurable: three spatial coordinates (x, y, z), time of exposure (t), excitation and emission wavelengths (L and l). In addition to imaging the fluorescence intensity, an add-on system can be used to characterize the lifetime of fluorescence in the specimen on the pixel-by-pixel basis using the time-domain fluorescence lifetime imaging microscopy (FLIM).

Additional features of the SP8 microscope include autofocusing, which keeps the position of the focal plane fixed relative to the coverslip surface over extended periods of time, detector electronic gating, which enables to delay detection relative to illumination for several nanoseconds thereby suppressing parasite reflection from the glass surface, and a resonant scanner, which enables fast scanning over 10 thousand lines per second. The system is also equipped with a stage-top incubator for live cell microscopy capable of maintaining appropriate temperature and atmosphere for experiments with living cells, and with an automatized microscope stage with the specimen holder that enables experiments that repeatedly visit multiple positions in the specimen over several hours.

Keywords: confocal microscopy; optical sectioning; microspectrofluorimetry; fluorescence lifetime imaging.

Table 1. Comparison of major features between the instrument configurations purchased in 2003 (Leica TCS SP2 AOBS) and the one purchased in 2013 (Leica SP8 X FLIM).

Feature	SP2(2003)	SP8(2013)
Scanning speed	1000 lines/second	1800 lines/second
Resonant scanner	—	12000lines/second
Excitation lines	8 discrete lines: 405, 458, 476, 488, 514, 543, 594 and 633 nm	5 discrete lines (405, 458, 476, 488 and 514 nm) and a continuous spectrum between 470 and 670 nm
Maximalimage size (pixels)	4096 × 4096	8192 × 8192
Maximal field of view	12 mm	22 mm
Detector gating	none	available for 2 detectors
Autofocusing	none	hardware and software autofocus options
Water-immersion objective	none	one
High content screening	none	available
FLIM	no	availablefor 2 channels

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P53/P63/P73 PROTEIN NETWORK IN HUMAN TUMORS

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The p53 tumor suppressor protein is critical in the cell growth control and the maintenance of genomic stability. Its activities are due, at least in part, to

the ability to form tetrameres that bind to specific DNA sequences and activate transcription of target genes. After discovery of p63 and p73, the p53 family members, it became evident that to understand the p53 pathways, all of them have to be taken into account. p73 and p63 were shown to activate many p53 target genes, both are able to induce cell cycle arrest and apoptosis and are activated upon DNA damage. They have important roles in carcinogenesis and during development. *TP63* and *TP73* generate transactivating forms (TAp73) as well as a number of N-terminally truncated transactivation-deficient isoforms (ΔNp73/ΔNp63) which can act as potential transdominant inhibitors of transactivation competent isoforms (TAp73, TAp63and p53). *TP63* and *TP73* genes are rarely mutated but frequently overexpressed in human tumors. *TP53* also has a second promoter P2,alternative translation initiation sites and undergoes alternative splicing, therefore generates multiple isoforms: p53α, (also named full-length p53, FLp53, canonicalp53 or TAp53α), p53β (or p53i9), p53γ, Δ40p53α, (or ΔNp53, p44 or p47), Δ40p53β, Δ40p53γ,Δ133p53α, Δ133p53β, Δ133p53γ, Δ160p53α,Δ160p53β and Δ160p53γ (*Figure 1*). p53 isoforms might play important roles in carcinogenesis and cancer treatment.

Since some mutant p53 proteins and ΔNp73 isoforms form heterocomplex with TAp73, we studied whether p53 isoforms can do the same and potentially act as dominant-negative inhibitors of TAp73. It has already been found that some isoforms form complex with wtp53 and some of them inhibit p53 tumor suppressor functions. Therefore, we studied the complex formation and co-immunoprecipitation assays showed that all p53 isoforms examined (p53β, p53γ, Δ40p53α, Δ133p53α, Δ133p53β and Δ133p53γ) can form complex with TAp73β. In order to analyze the effect of isoforms on TAp73β activity, we performed reporter assay using natural promoter with the p73/p53 binding site driving the luciferase reporter. All examined isoforms counteract TAp73β transactivation function but with different efficiency and in a promoter-dependent manner. Furthermore, we assessed the apoptotic activity of TAp73β when coexpressed with several p53 isoforms. Percentage of apoptotic cells was determined after Annexin V and propidiumiodide staining by FACS analysis. Interestingly, apoptotic activity of TAp73β was augmented by co-expression of p53β, while Δ133p53α and β inhibit its apoptotic activity most efficiently. Next, the half-lives of different p53 isoforms has been determined showing that p53γ isoform has the shortest while Δ133p53γ has the longest half-life.

Additionally, we have studied whether some mutant p53 (R175H, L194F, R280K, R282W) can form complex with TAp73β and TAp63α and potentially act

as dominant negative inhibitors. Co-immunoprecipitation assay and very sensitive proximity ligation assay (PLA) confirmed that mutant p53 can form complex with TAp73β and TAp63α. Common polymorphism 72Arg binds more efficiently to p63 and p73 than the equivalent 72Pro. The results of luciferase reporter assays have shown that all mutant p53 inhibit transcriptional activity of TAp73β but with different efficiency. Furthermore, mutant $p53^{248}$ and $p53^{280}$ are the most efficient inhibitors of TAp73β apoptotic activity of all tested mutant p53.

Defining the interactions between p53/p63/p73 would gain insight into how the mutant p53 and p53 isoforms modulate the functions of p73 and p63 in tumorigenesis. Also, deeper understanding of p53 protein network may guide decision on therapeutic approach and may provide prognostic information in cancer biology.

Keywords: p53; p63; p73; carcinogenesis; protein interaction.

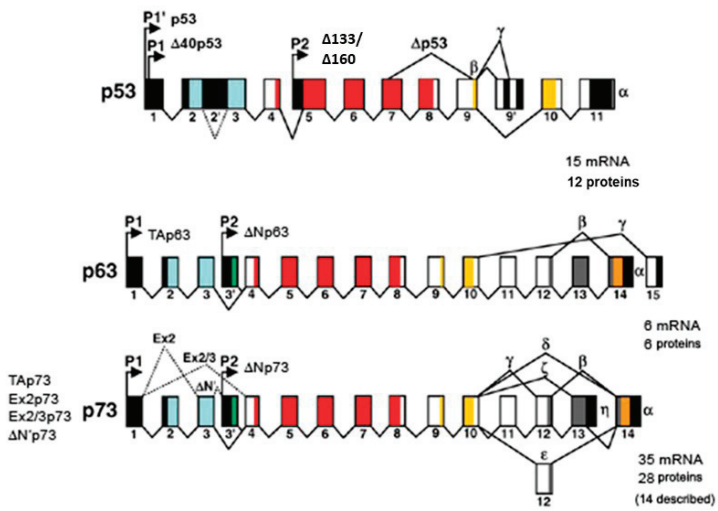

Figure 1. The structure of human p53, p63 and p73 genes. Alternative splicing and alternative promoters (P1 and P2) are indicated.

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CELL BLOCK – VIEW BELOW MORPHOLOGY LEVEL

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The cell block method is a procedure of preparing a cytological sample that can be analyzed using pathohistological methods. Historically, the first attempts to create a cell block date back to the 1880s. Throughout history, numerous methods of building a cell block have been developed that we have at our disposal even today. At the Department of Pathology and Clinical Cytology of Clinical Hospital Rijeka, the most used method to build cell blocks is agar method. It has been shown that we have achieved optimal results using this method. It should be noted that the classical cytological material is suitable for all types of morphological, immunocytochemical and molecular analyses; however, most of the present-day immunoassay tests for predictive markers are validated on histological material, which, although much more representative, currently limits the use of cytological material.

The cell block method is rarely used alone for itself, meaning that it is only used to evaluate morphology alone (*Figures 1 and 2*). Still, there are studies that have shown the diagnostic advantage of this method in cytology, such as tumor classification, owing to its ability to have insight into tumor architecture. Since cell blocks behave as small biopsies, they can be used for morphological studies of apoptosis evaluation on standard HE preparations or by using a TUNEL method that provides visualization of specific DNA fractures due to the apoptosis process. Immunohistochemistry can be used to analyze the presence of different proteins due to the dysregulation of the apoptosis process in cells encapsulated in a cell block, and their detection serves simultaneously for diagnostic purposes of distinguishing precancerous conditions and benign and malignant tumors. In general, studies evaluating the presence of various apoptosis markers in cell block material are extremely rare and have been appearing just recently.

The disadvantages of agar-method compared to classical cytological samples are less pronounced cellularity resulting from initially scarce cellular material or loss of material during processing, which reduces the representativeness of the sample itself to the observed process. Although the cell blocks generally

obtain enough cells of well-preserved morphology (*Figure 3*), sometimes the cells are damaged by thermal treatment alone. The main advantage of using cell blocks is the ability to analyze different immunohistochemical parameters in standardized and automated systems using tissue samples along with cell block samples for positive and negative control (*Figure 4*).

In conclusion, although the cell block method has been present over a century, a major shift in its application has occurred over the last 15 years due to the need for standardization of cytology diagnostics, as well as the assessment of prognostic and predictive tumor factors in patients where only cytological material is available.

Keywords: apoptosis; cell block; prognosis; standardization; tumor.

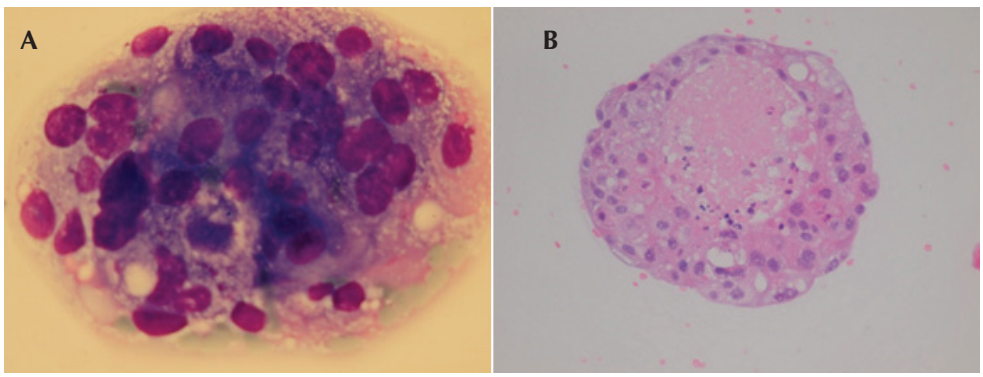

Figure 1. Squamous cell carcinoma in pericardial effusion. A. Pericardial effusion sediment, MGG. B. Cell Block, HE.

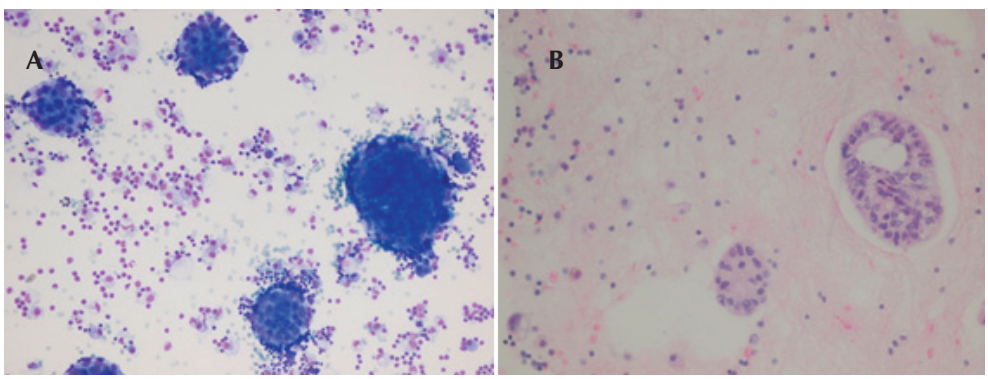

Figure 2. Breast adenocarcinoma in pleural effusion. A. Pleural effusion sediment, MGG. B. Cell Block, HE

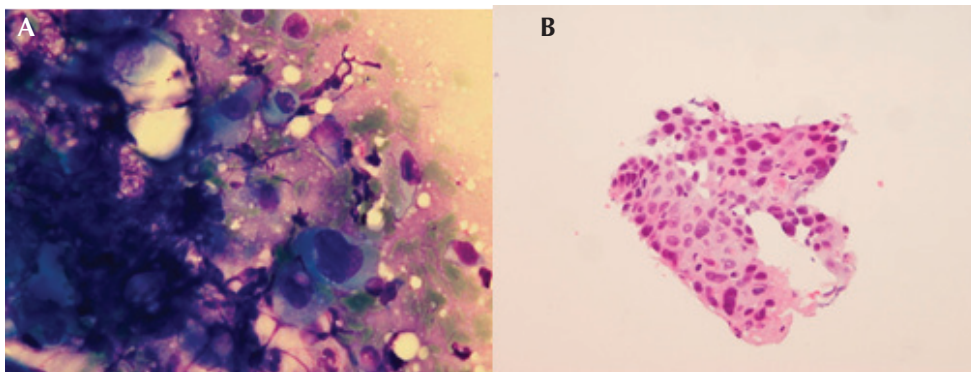

Figure 3. Squamous cell lung cancer. A. Bronchial brush smear, MGG. B. Cell Block, HE

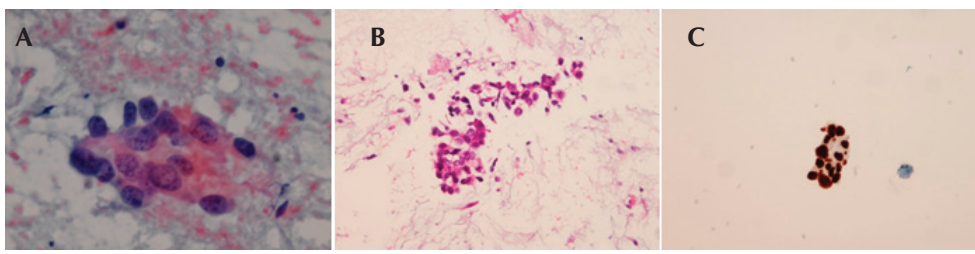

Figure 4. Lung Adenocarcinoma. A. Bronchial brush smear, PAPA. B. Cell Block, HE. C. Cell Block, immunohistochemical analysis by TTF-1 marking.

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VALPROATE ENHANCES APOPTOSIS OF GASTRULATING MAMMALIAN EMBRYO CULTIVATED IN VITRO

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Valproate is a known antiepileptic responsible for the valproate syndrome (FVS) in children whose mothers have used it during pregnancy (1). In 2014 The European Medicines Agency warned against the use of valproate in treating epilepsy but also some other illnesses such as e.g. the migraine (2). A recent study has described 29 cases of FVS diagnosed in Ireland from 1995-2016. Among malformations were those of the neural tube, heart, limbs, craniofacial etc. (2). However, all effects are still not completely explained at the molecular level (3,4).

Today it is clear that anomalies of development of specific organs are induced at the specific time-windows or critical phases that correlate with the phases of organ development (5). On the basis of his experiments with various teratogenic agents, the founder of the Zagreb School of Mammalian Embryology academician N. Škreb (5) has proposed an "all or none" hypothesis regarding the early postimplantation development at the Pallanza conference already in 1960 (6). Before the formation of the mesoderm, the young mammalian embryo is either destroyed by the teratogen or recovers completely, and therefore these early stages are not susceptible to teratogens (5). The formation of the mesoderm and the three definitive germ-layers takes place at gastrulation. Therefore, gastrulation is the first and the most critical phase of mammalian development where the early embryo loses its plasticity. At that stage the „all or none rule" is lost and the influence of a teratogenic agent may be visible after birth (5,6,7).

In this study we aimed to investigate the direct effect of valproate on the development of the gastrulating rat embryo (embryo proper) devoid of its extraembryonic parts and outside of the maternal environment.

Fischer rat embryos were microsurgically isolated and grown in the original organ-culture model with the Eagle's Minimum Essential Medium and 50% rat serum (controls). Valproate (2mM or 1mM) was added to the culture medium and cultures were grown for 3 days or 2 weeks with 5% CO_2 in ahumidified incubator. We used immunohistochemistry to detect cleaved caspase 3 as the marker of apoptosis and the Proliferating Cell Nuclear Antigen as the marker of proliferation. Stereology was used to calculate the volume density (V_v) of the apoptotic marker and proliferation index was calculated. Growth was measured by an ocular micrometer. Acetylated histone expression was detected by the Western blot.

By measuring overall growth, from the third day onwards, a significantly lower growth was discovered in cultures with valproate that was also lower in embryos treated with the higher dose. After three days of culture, in embryos treated with the higher dose of valproate, a significantly higher apoptotic activity (Vv of cleaved caspase-3) was discovered than in controls and in embryos treated with the lower dose of valproate (*Figure 1*). After two weeks in culture, the proliferation index (PCNA index) was significantly lower in embryos treated with a higher dose of valproate. Higher dose also inhibited differentiation, especially of the nervous tissue. Higher expression of the acetylated histone H3 was discovered in embryos treated with valproate.

It can be concluded that valproate negatively affects developmental processes already at the gastrulation stage, and its impact is dose-dependent. Apoptosis enhanced by the higher dose (2mM) may be connected to the enhanced histone acetylation caused by valproate that is an epigenetic drug and inhibitor of the histone deacetylase (3).

Keywords: apoptosis; valproate; embryo; gastrulation; *in vitro*.

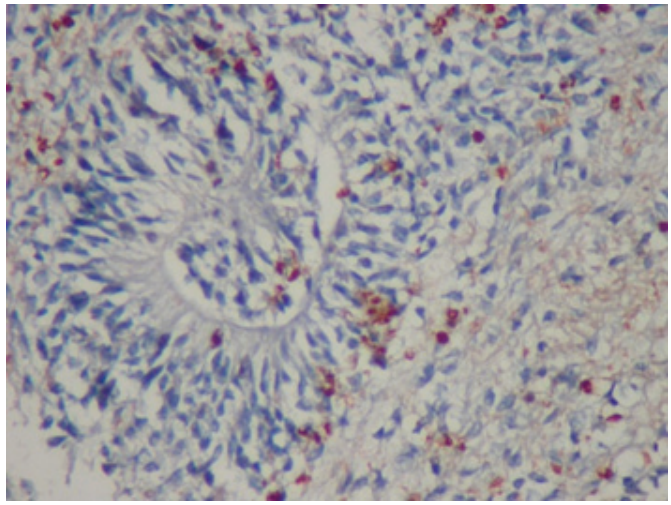

Figure 1. Cleaved caspase 3 in the neuroepithelium of cultivated embryos treated with 1 mM VPA.

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