

**„VICTOR BABEȘ” UNIVERSITY OF MEDICINE AND PHARMACY
TIMIȘOARA
FACULTY OF MEDICINE
Department IX**

GURAN KRISTINE



**CURRENT ASPECTS RELATED TO PRECLINICAL
METHODS, THERAPEUTIC PROTOCOL AND
PREVENTION IN NAZOPHARINGIAN CARCINOMA**

ABSTRACT

Scientific Coordinator

PROF. UNIV. DR. POENARU MARIOARA

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ABSTRACT

The research topic is based on the understanding of the mechanisms associated with squamous cell carcinomas in the ENT field. Despite the daily progress at both diagnostic and therapeutic protocol levels, the mechanisms involved in malignant processes remain incompletely understood and survival rates in certain malignancies have not improved significantly in the last three decades.

Squamous cell carcinoma is an epithelial malignancy that occurs in organs that are normally covered with squamous epithelium (e.g., skin, mouth, lips, esophagus, prostate, lungs, etc.). The most common cases of squamous cell carcinomas are found in malignant diseases of: skin - non-melanoma cancers, head and neck, esophagus and lung. Given the multitude of tissues in which squamous cell carcinoma can occur and develop, it can be said that it holds the first place globally in terms of diagnosis.

The therapeutic approach applied to squamous cell carcinoma is varied, it can be simple or combined, but the beneficial results for the patient are delayed and the costs related to the medical care of a patient with squamous cell carcinoma can be relatively high. A number of genetic changes have been identified in squamous cell carcinoma subtypes, but the molecular mechanisms that contribute to tumor initiation and progression are still far from being fully elucidated.

Squamous cell carcinomas show a number of phenotypic and molecular characteristics that make new markers or drug targets, intended to discover or even obtained, in certain types of individual squamous cell carcinomas can help develop new diagnostic and treatment protocols.

This paper is a topical and interesting one, mainly addressing nasopharyngeal carcinoma. Squamous cell carcinomas of the head and neck represent the vast majority of head and neck cancers and rank in the top ten most common malignancies diagnosed annually worldwide. Squamous cell carcinomas of the head and neck are a group of tumor diseases that occur from the surfaces of the squamous mucosa - oral cavity, nasal cavities, paranasal sinuses, nasopharynx, oropharynx, hypopharynx and larynx. In contrast to their declining overall incidence, which is mainly due to preventive actions (such as smoking cessation, careful oral hygiene, etc.), nasopharyngeal carcinoma has an increasing incidence, especially among young people, which suggests that some less common behavioral and environmental factors play a key role in its epidemiology. Even though, in

recent years, a number of progressess have been made in the detection and treatment applied, the vast majority of patients have metastatic diseases at the time of diagnosis, which reduces the overall 5-year survival rate to an extremely low percentage. This late diagnosis, along with the formation of additional primary tumors and metastases contributes greatly to this low survival rate.

Nasopharyngeal carcinoma, especially of the undifferentiated subtype, remains endemic in southern China (where the maximum annual incidence is around 30 per 100,000 people). The overall prognosis has improved markedly in recent years, mainly due to advances in management, but also due to improved radiotherapy technology (the gold standard for first-line treatment), wider application of chemotherapy and more accurate staging of the disease. Current therapeutic protocols depend on the stage of the disease and it is generally accepted that stage I be addressed by radiotherapy, stage II by radiotherapy with or without chemotherapy and advanced stages, III and IV, by radiotherapy combined with chemotherapy. In most cases, an excellent local control can be achieved, but the failure is represented by distant metastases, the treatment results in these cases being very poor. To all this are added the problems related to the toxicity of therapeutic protocols, both radiotherapy and chemotherapy being known for the toxic side effects associated with them. Due to the aforementioned impediments, the therapeutic approach to nasopharyngeal carcinoma, registered in recent years, especially in young people, remains a challenge for all specialists in the field at local, national and global level, being very important interdisciplinary collaboration.

At the same time, few, incomplete and interpretable data regarding the occurrence, development, diagnosis and treatment of nasopharyngeal carcinoma, led to the establishment for the present paper of the analysis of these aspects in order to contribute to updating data and understanding certain mechanisms involved in associated malignancies.

Therefore, the main objectives established for the present thesis were:

- 1) Deepening preclinical methods for the evaluation and prevention of squamous cell carcinomas of the head and neck
- 2) Evaluation of chemotherapy, intestinal processes and alternative therapies
- 3) Analysis of the incidence of nasopharyngeal carcinoma in Romania and correlation with different factors of occurrence and prevention

This paper is structured, according to the methodological norms in force, on two main parts, the general part and the special part, which continues with the sub-chapter of

conclusions and personal contributions, followed by the current bibliography studied, which also includes publications associated with own research. .

In the general part, the first chapter addresses aspects related to nasopharyngeal carcinoma: (1) generalities, current perspectives, (2) epidemiology - incidence and mortality, (3) risk factors, (4) pathological aspects and (5) current therapy. The second chapter of the general part presents the involvement of bacterial infections in the diagnosis and treatment of cancer with details on the oral microbiota and the intestinal microbiota.

The special part contains three main chapters. The first chapter presents the personal contributions made following experimental achievements related to preclinical methods for the evaluation and prevention of squamous cell carcinomas of the head and neck. Chemotherapeutic protocols are commonly applied in the treatment of cancer. However, chemotherapy is not without its drawbacks, as its therapeutic benefits are generally accompanied by side effects categorized as toxic. The possibility of obtaining new therapeutic agents with as few side effects as possible, which can improve the quality of life of patients during chemotherapy, is currently being intensively studied globally. Herbal medicines have been widely approved as an alternative or adjunct to conventional chemotherapy in the fight against cancer, with a number of herbal bioactive compounds being identified annually. Unfortunately, there is evidence that reveals the limited efficacy of monotherapy using compounds derived from natural sources in the prevention and treatment of cancer. Therefore, the use of natural compounds, plant extracts as mixtures of natural compounds that act synergistically has become a promising option. Moreover, plant extracts or their main components can be co-administered with conventional chemotherapeutic agents, combating the development of drug resistance and toxic effects, such as nausea and vomiting. One of the plants that attracted the attention of researchers in the field is *Melissa officinalis* L. It is also known as lemon balm, being a medicinal plant belonging to the family Lamiaceae. The medicinal plant is well-known for its wide range of therapeutic effects such as: sedative, antimicrobial, spasmolytic, antioxidant, etc. These effects are determined by its biologically active constituents such as flavonoids, polyphenolic compounds, triterpenes, sesquiterpenes and tannins. Its benefits have already been recognized in the prevention of oral diseases (e.g., effectiveness in combating dental plaque) and gastrointestinal diseases (e.g., irritable bowel syndrome). At the same time, several studies confirm that *M. officinalis* extracts cause a strong antiproliferative activity on different types of cancer. However, data on the anticancer potential of *M. officinalis*

extracts against oropharyngeal and colorectal squamous cell carcinomas are quite rare. Taking into account the aforementioned data, the research study proposed the main aim of determining the pharmacological profile of the total extract of *M. officinalis* (MOte) obtained from leaves as a potential alternative for the prophylaxis of oral, pharyngeal and colorectal carcinoma. In this regard, the following objectives were pursued: (i) characterization of the total extract of *M. officinalis* in terms of chemical constituents - it is known that each extract has its own composition that varies according to a number of factors related to the environment and mode of extraction and separation, mainly; (ii) evaluation of the antioxidant capacity of MOte - a good antioxidant activity leads to the control of reactive oxygen species; (iii) investigation of the antimicrobial activity of MOte; (iv) evaluation of the cytotoxic potential of MOte in human squamous cell carcinoma (using FaDu and SCC-4 cell lines) and colorectal adenocarcinoma (using Caco-2 and HT-29 cell lines) compared to healthy cells (PGK- primary human gingival keratinocytes) and (v) control of the impact of MOte on the process of angiogenesis *in ovo*. The results of this study provide consistent evidence that the total extract of *Melissa officinalis* leaves has multiple benefits (antioxidant, antimicrobial, antiangiogenic and cytotoxic effects) as a potential agent for the chemoprevention of tongue, pharyngeal and colorectal cancers. The main hypothesis was based on the fact that the pharmacological profile of MOte is recognized, this plant can be considered a potential candidate for the prophylaxis of these cancers, because it suppresses cell proliferation and angiogenesis, acting as an antioxidant and antimicrobial agent. The main findings in this regard are: (i) MOte significantly reduced the viability of pharyngeal and tongue squamous cells, as well as the viability of colorectal carcinoma cells in a concentration-dependent trend, the highest sensitivity being observed in the case of FaDu; (ii) at the highest concentration tested (100 ug / mL), MOte regressed angiogenesis; and (iii) MOte caused a dose-dependent antibacterial effect on Gram-positive and Gram-negative bacteria. However, further studies (e.g., *in vivo* clinical trials) are needed to confirm the usefulness of the total *M. officinalis* extract in preventing the development of oral, pharyngeal and colorectal neoplasms.

The second chapter of the special part deals with aspects related to chemotherapy, processes that take place in the intestine and shows the experimental part associated with alternative therapies. Squamous cell carcinomas of the head and neck are relatively common and are usually associated with radical surgery and poor results, with a poor prognosis in those with advanced or metastatic disease. On the other hand, in the case of

nasopharyngeal carcinoma there is a good survival rate even with non-surgical treatment, under certain conditions. The incidence of metastases is often underestimated with clinical diagnosis, with several factors influencing the incidence of distant metastases (e.g., location of primary tumor, initial T and N stage of the neoplasm, and the presence or absence of regional control over the clavicle). Nasopharyngeal carcinoma is known for its propensity for both lymphatic and hematogenous spread. Colorectal metastasis has been observed rarely in patients, demonstrating awareness of this entity. Cancers involving the bowel are usually primary cancers of the large or small intestine, have a direct invasion of primary cancers that occur from other adjacent abdominal organs or peritoneal carcinomatosis. Due to the uniqueness and rarity of the association between nasopharyngeal carcinoma and metastasis to the colon, it is important to consider whether the colorectal lesion is a metastatic lesion or a new primary tumor. It is estimated that up to ~ 28% of patients with nasopharyngeal squamous cell carcinoma will develop distant metastases.

Thus, it is important that in clinical practice, specialists pay special attention to patients with gastrointestinal disorders, regardless of the state of remission of squamous cell carcinoma. Physicians need to be aware of the high-risk characteristics of the primary tumor, the limitations of investigations, and the concomitant presence of other distant metastases, which can drastically change treatment and outcome. Survival rates in non-primary colorectal carcinoma vary due to the rarity of the entity, different types of primary tumors, and tumor stages. Currently, although radiotherapy has a palliative role in bone and occasionally lung and brain metastases, it does not appear to play a decisive role in adjuvant treatment (chemotherapy or radiotherapy) in colorectal metastases, and median survival is short, ranging from 3 at 5 months. Therefore, future strategies may further deepen the role of microbes in the development of colon metastases. Ionic liquids are a modern conceptual alternative due to their widespread use in major areas of research. For example, these liquids can be used as: (i) alternative solvents to volatile organic compounds, (ii) media for electrodeposition, (iii) catalysts and biocatalysts, (iv) potential corrosion inhibitors, or (v) solvents in the food industry, having several key physicochemical properties for such applications, including high thermal stability, high ionic conductivity, low vapor pressure, non-flammability, strength and high polarity. As a result, they have attracted a growing interest in medical research. Ionic fluids can affect biological systems at various biochemical levels, from simple macromolecules to complex

metabolic mechanisms in prokaryotic and eukaryotic cells. In this context, research on the antimicrobial activity of different classes of ionic liquids on microorganisms of clinical and environmental importance has emerged as an important research direction in recent decades. Recent studies have shown that many types of ionic liquids have the potential to inhibit the growth of various bacteria and fungi, making them useful for various applications in medicine and industry. It has also been reported that antimicrobial activity is related to the length of the substituent chain, noting that short-chain compounds exert a lower antimicrobial efficacy compared to long-chain compounds. This antimicrobial effect has been studied on both standardized bacterial strains and bacterial strains isolated from the hospital environment and it has been found that some of the strains of interest are resistant to antibiotics and are affected by these types of compounds. However, further studies are needed to fully assess the clinical applicability of ionic liquids, an important topic being to decipher their toxicological, antimicrobial and antiproliferative effect. The cytotoxicity exerted by ionic liquids was extensively analyzed using *in vitro* 2D monolayer culture systems and various cell lines as study systems - both normal cells (fibroblasts, osteoblasts, macrophages, keratinocytes, rat glial cells) and neoplastic cell lines (breast, colon, lung, liver carcinoma and leukemia), data found in the literature.

The present study was conducted to investigate the antibacterial and antiproliferative effect of the ionic liquid tetrahexylammonium bromide (THABr) formula. The following standardized bacterial strains were used to evaluate the antibacterial effect of THABr_LI: (a) Gram-positive (Gram +) bacteria - *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Enterococcus faecalis*; and (b) Gram-negative (Gram-) bacteria - *Escherichia coli*, *Salmonella typhimurium*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Haemophilus influenzae*.

The Caco-2 cell line (colorectal adenocarcinoma) served as a study system and was cultured *in vitro* using both 2D and 3D culture systems. At the time of study design, *in vitro* cytotoxicity research for ionic liquids appears to have been performed using only 2D cell cultures. This is therefore the first study to use 3D cultured cells to study the cytotoxic effect of ionic liquids. Using a comparative approach, the present study also extends, in a bidirectional way, previous knowledge about the potential clinical significance of these compounds. The biological activity of ionic liquids depends primarily on their state of hydration, although their mechanisms of action may vary between different organisms, water being essential for all living systems. Therefore, the main factors that modulate the

biological activity of ionic liquids are their solubility and interaction with water. The antibacterial effect of THABr ionic liquid varies depending on the concentrations tested and the bacterial strain studied, in general, the effect is highlighted when testing the highest concentrations. In the case of Gram + bacteria, the sensitivity to the ionic liquid tested decreases as follows: *S. aureus* > *S. pneumoniae* > *E. faecalis*. In Gram- bacterial strains, the antibacterial effect of ionic fluid decreases as follows: *H. influenzae* > *P. mirabilis* > *P. aeruginosa* > *K. pneumoniae* > *E. coli* > *S. typhimurium*. Regarding the cytotoxic potential on Caco-2 neoplastic colon cells, moderate results were observed. Future studies should be performed to evaluate the impact of THABr_LI on three-dimensional aggregation of tumor cells.

The last chapter of the special part describes the incidence of nasopharyngeal carcinoma in the western part of our country, and a series of correlations are made with different factors of appearance (role of food and causes) but also of prevention (importance of oral hygiene and natural resources). Dedicated studies reveal that the mutational context of nasopharyngeal carcinoma in Southeast Europe seems to have some similarities and important differences compared to models in Southeast Asia. The prevalence of presumed pathogenic somatic BRCA1 mutations and the accompanying degree of genomic instability in European nasopharyngeal carcinoma appear to be inclined towards alternative treatments. It has also been reported that genetic modifications of morphologically normal immune cell infiltrates of nasopharyngeal carcinoma can be used as observational data for basic research studies on viral carcinogenesis and on the interference of the tumor microenvironment with the biological characteristics of nasopharyngeal carcinoma. In Romania, it was considered that the late diagnosis of nasopharyngeal carcinoma may be due to misdiagnosis, but also due to poor medical education of the population. At the same time, the poor quality of medical equipment and sometimes the superficiality with which the patient is treated, can be considered two other causes of the advanced diagnosis of nasopharyngeal carcinoma registered in our country.

The main purpose of this study was to evaluate the incidence of cases of nasopharyngeal carcinoma diagnosed in western Romania and the role of diet in its development, the importance of medicinal plants with anticancer properties and their bioactivity in squamous cell carcinoma. Therefore, the cases of nasopharyngeal carcinoma in western Romania were analyzed and, according to the expected data, it was shown that men are more susceptible to this form of shared carcinoma with women. Of the cases diagnosed

with nasopharyngeal carcinoma, 20% belong to women and the remaining 80% to men. A large majority of the patients studied in this case had microbial infections. The predominantly identified bacterial cultures were both gram-positive and gram-negative or fungal. The species mainly identified in males were: *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus maltophilia*, *Candida glabrada*. In the case of females, the predominantly identified bacteria were: *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Candida albicans*.

The relatively small number of cases encountered in western Romania during six years of evaluation, is a gratifying proof that the European population has an unfavorable genome for nasopharyngeal carcinoma. The correlation between lifestyle and the development of nasopharyngeal carcinoma is still deficient and poorly understood. For example, smoking, the use of herbal nasal sprays, or the use of excess tea have been associated with nasopharyngeal carcinoma. European lifestyle and eating habits discourage the activation of this type of cancer. Foods that contain N-nitroso compounds, which are DNA destroyers, can accelerate the onset and development of the disease. Fortunately, in the Balkans, foods rich in nitrogen-based carcinogens are significantly less common compared to the pandemic area of China. The treatment of the disease involves primarily irradiation and, if necessary, the combination with chemotherapeutics. These procedures are accompanied by a number of significant side effects. Side effects can be alleviated by adopting a balanced diet and a carefully implemented lifestyle.

The natural antioxidants found in fruits, vegetables, certain teas, and wine, are healthy and safe solutions to combat pro-inflammatory diseases that lead to the development of malignant processes. Polyphenolic compounds have an activity of reducing the number of cancer cells that must be deepened in order to establish beneficial protocols for the chemoprevention of malignant processes. Plant extract refers to a type of substance extracted or processed in whole or in part from the plant by appropriate solvents or methods. However, choosing the right plant extract has become an urgent issue and needs to be resolved clinically. Classifying the likelihood of advantages and disadvantages based on different outcome indicators can help clinicians choose the best scheme from different intervention measures.

At the end of the special part, the conclusions and own contributions are presented clearly and succinctly.

The research methods adopted to achieve the proposed objectives are current and the data obtained and presented both in this paper and in specialized publications bring a plus in more detailed knowledge of how the manifestation and approach of nasopharyngeal carcinoma.

The scientific research objectives set out in this paper have been successfully completed.

There is currently a need to improve individualized therapy through better precision in the selection and administration of treatments, to improve the integration of multimodal therapies and interdisciplinary cooperation and inter-institutional collaboration. Emphasis should be placed on promoting interdisciplinary collaboration within and between institutions, at local, national and international levels, in the development of new nasopharyngeal carcinoma models along with high quality screening of new compounds with relevant clinical and preclinical study designs. The concept of individualized therapy also applies to the optimal selection of patients for adjuvant chemotherapy during radiation therapy, and multinational studies now address the role of adjuvant chemotherapy in different types of subpopulations.

Therefore, better designed multi-institutional studies are needed, and this requires a collective effort between expert centers in harmonizing and standardizing procedures related to diagnosis, therapeutic protocol and especially prevention taking into account the early age at which this disease is diagnosed.