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PhD THESIS

DIAGNOSIS, TREATMENT, AND PROGNOSIS OF HPV-POSITIVE OROPHARYNGEAL CANCER

- A B S T R A C T -

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Motivation for thesis

This doctoral thesis focuses on the clinical, immunohistochemical, and therapeutic aspects of oropharyngeal squamous cell carcinomas (OPSCC) in relation to Human Papillomavirus (HPV) infection. The motivation for choosing this research topic stems from the significant rise in the number of patients diagnosed with oropharyngeal cancer and the highly variable progression of each case. Despite advancements in medical science, there is still no well-established treatment algorithm for these patients, prompting the need for further research. The primary aim is to identify new clinical, immunohistochemical, therapeutic, and prognostic parameters to optimize the management of OPSCC in relation to HPV. Additionally, the study explores new treatment methods for oncology patients, including the application of novel medications to those suffering from oropharyngeal cancer. This topic is of great importance due to the increasing number of patients requiring medical care and their inconsistent responses to radiotherapeutic treatment.

General Part

HPV and Oropharynx

Oropharyngeal cancer presents a complex pathology with various clinical and molecular dynamics that require in-depth exploration. The connection between HPV, particularly HPV 16, and oropharyngeal cancer has been studied extensively, revealing the crucial role of p16 overexpression. P16, a cyclin-dependent kinase inhibitor, serves as a surrogate marker for HPV-positive cancers and is often associated with a favorable prognosis and therapeutic response. This thesis focuses on analyzing the implications and mechanisms of p16 overexpression and HPV status in oropharyngeal cancers, aiming to understand how these factors influence the disease's molecular characteristics and clinical progression. The research also investigates how the interaction between p16 overexpression and HPV status affects radiosensitivity and determines the response to various therapeutic approaches. Utilizing statistical analyses, this study aims to bridge immunohistochemical findings with clinical applications, ensuring that molecular and clinical discoveries translate into practical implications for patient treatment plans and disease progression.

Furthermore, the research evaluates a novel drug delivery mechanism utilizing curcumin, a natural compound with known anti-inflammatory, antioxidant, and anticancer properties. The study assesses the safety and efficacy of this delivery system in patients with oropharyngeal cancer. Ultimately, the thesis aims to link clinical and management aspects of oropharyngeal cancer by focusing on HPV status and p16 overexpression, exploring the disease's complex molecular and clinical pathways. This research provides detailed insights and practical tools to improve understanding, management strategies, and optimize patient outcomes and quality of life for individuals with oropharyngeal cancer.

Anatomical particularities of the Oropharynx in relationship to cancer

The oropharynx is a crucial component of the human upper aerodigestive tract, responsible for various functions such as breathing, swallowing, and speech production. The region is located in the center of the cervical area, posterior to the oral cavity, and serves as a passageway between the nasopharynx above and the hypopharynx below. The oropharynx includes several critical anatomical regions, each playing a distinct role in cancer development and treatment. The base of the tongue, rich in lymphoid tissue, plays a significant role in the immune defense system by detecting and responding to pathogens. The palatine tonsils, encapsulated by a mucous membrane and containing numerous crypts, contribute to the immune system by producing lymphocytes and generating an antibody-mediated immune response. The soft palate, comprising muscle fibers covered by a mucous membrane, is involved in speech and swallowing. The posterior and lateral oropharyngeal walls, although rare cancer sites, are composed of pharyngeal constrictor muscles, connective tissue, and a mucosal layer, contributing to their functionality and health.

The base of the tongue contains lymphoid tissue, which forms part of the lingual tonsils. This tissue contributes to the immune defense system by helping to detect and respond to pathogens. This area is supplied by blood vessels and nerves, including the glossopharyngeal nerve (cranial nerve IX) and branches of the vagus nerve (cranial nerve X) responsible for taste and sensation. Its main function is to push down the food in coordination with other structures of the pharynx into the oesophagus during swallowing. It moves upwards and backwards to help close the nasopharynx, transforming it into a cavity and preventing food from entering the nose. Cancer of the base of the tongue remains one of the most problematic oropharyngeal sites, remarkably so by its dysfunctional post-surgical remanent. Thus, a significant improvement remains in radiotherapy with favorable outcomes, especially regarding HPV infection and p16 overexpression; some studies suggested survivability rates as high as 92% in HPV positive versus less than 75% in negative HPV.

The palatine tonsils are a pair of oval-shaped lymphoid structures in the oropharynx, situated in the tonsillar fossa between the palatoglossal and palatopharyngeal arches. Each tonsil is encapsulated by a mucous membrane and contains numerous crypts or invaginations, which increase the surface area for antigen exposure and trapping of pathogens. Functionally, the palatine tonsils play a crucial role in the immune system, particularly in producing lymphocytes and generating an antibody-mediated immune response. They germinal centers where B cells proliferate, differentiate, and mutate to produce high-affinity antibodies essential for adaptive immunity. Activated B cells become plasma cells, releasing antibodies into the bloodstream and mucosal surfaces, and some become memory B cells for long-term immunity. Helper T cells (CD4+ T cells) in the tonsils activate B cells and cytotoxic T cells (CD8+ T cells) to kill infected cells, while regulatory T cells modulate the immune response. Tonsils are major sites for producing secretory IgA (sIgA), which binds to pathogens, preventing their adherence and penetration of epithelial cells, thus neutralizing them. However, the crypts of the palatine tonsils can harbor debris, including food particles, dead cells, and bacteria, making them susceptible to infections, including HPV, and thus rendering the swab test inaccurate compared to

cervical swabs. Tonsillar cancer is one of the fastest-growing types of cancer found in the Western world, especially due to its HPV etiology, and second only to laryngeal cancer. It is still caused by classic factors such as smoking and alcohol consumption. It responds relatively well to chemotherapy and radiation if the linkage to HPV exists, even in more advanced forms with adenopathy or local invasion. Surgery remains the most important treatment in low-to-medium-stage stages, considering the adoption of more modern techniques like TORS or transoral laser microsurgery.

The soft palate is a flexible, muscular structure located at the back of the roof of the mouth, posterior to the hard palate. It comprises muscle fibers covered by a mucous membrane and plays a critical role in speech and swallowing. Anatomically, the soft palate consists of several muscles, including the tensor veli palatini, musculus uvulae, levator veli palatini, palatoglossus, and palatopharyngeus. These muscles are innervated primarily by the pharyngeal plexus, which receives contributions from the vagus nerve (cranial nerve X) and the glossopharyngeal nerve (cranial nerve IX). The blood supply to the soft palate comes from the ascending palatine artery, a branch of the facial artery, and the lesser palatine arteries, branches of the maxillary artery. The soft palate is among the rarest sites of carcinogenesis but is important nonetheless. Cancer of the soft palate represents almost 2% of head and neck mucosal malignancies, with more than 50% being squamous cell carcinomas. Other types include adenocarcinomas, adenoid cystic carcinoma, mucoepidermoid carcinoma, or anaplastic carcinoma. A particular challenge with soft palate cancer is that it is sometimes misdiagnosed when the tumor forms on the nasal surface. A comprehensive endoscopic approach is necessary to fully visualize the entire soft palate for accurate diagnosis.

The posterior and lateral oropharyngeal walls are critical yet rarely affected regions in oropharyngeal cancer. The posterior oropharyngeal wall is the back part of the throat, forming the oropharynx's rear boundary, extending from the soft palate to the level of the cricoarytenoid articulation. It comprises the pharyngeal constrictor muscles, connective tissue, and mucosal lining. These muscles include the superior, middle, and inferior pharyngeal constrictors, which function together to propel food and liquids from the mouth to the esophagus during swallowing. The mucosal lining is rich in lymphoid tissue, contributing to the immune defense by detecting and responding to pathogens entering the oral and nasal cavities. The lateral oropharyngeal walls are lateral to the oropharynx, continuing from the soft palate to the upper edge of the epiglottis. These walls also comprise pharyngeal constrictor muscles, connective tissue, and a mucosal layer. Posterior and lateral pharyngeal wall squamous cell carcinomas are extremely rare, comprising only 12-20% of pharyngeal squamous cell carcinomas. Due to the relative lack of symptoms and the anatomical depth, these cancers might go unnoticed in the initial stages, potentially leading to a late-stage diagnosis. Managing posterior wall cancers requires careful consideration to safeguard nearby structures and maintain function, although patients might develop velopharyngeal insufficiency after treatment. The tumor's tendency to spread submucosally further complicates early diagnosis, often resulting in a late-stage diagnosis. Consequently, these cancers are associated with poor prognosis, with a 5-year survival rate under 30%.

HPV and Oropharyngeal Cancer

HPV, particularly HPV 16, significantly influences the pathogenesis of oropharyngeal cancers by disrupting cell cycle regulation through the degradation of tumor suppressor proteins like p53 and retinoblastoma protein (pRb). This disruption leads to uncontrolled cell proliferation. The thesis highlights the increasing incidence of HPV-positive oropharyngeal cancers, which respond better to treatment and have more favorable prognoses compared to HPV-negative cancers. HPV is a non-enveloped virus with a circular double-stranded DNA genome. It is classified into high-risk and low-risk categories based on their potential to cause cancer. High-risk HPVs, such as HPV 16 and HPV 18, are associated with malignancies, including cervical cancer, oropharyngeal cancer, and anal cancer. These types can integrate their DNA into the host cell's genome, disrupting cell regulatory mechanisms, and promoting uncontrolled cell division, leading to tumor formation. Low-risk HPVs, such as HPV 6 and HPV 11, typically cause benign warts and have a minimal risk of developing into cancer but are also involved in laryngeal pathologies.

The etiopathogenesis of oropharyngeal cancer related to HPV involves a complex interplay of viral actions at the cellular level that ultimately leads to malignant transformation. The most commonly implicated HPV strain in oropharyngeal cancers is HPV type 16, within most than 70-80% of cases compared to cervical cancer where it varies from 30-80%. The process begins when HPV infects basal epithelial cells of the oropharynx, particularly in areas like the tonsils and the base of the tongue, which are rich in lymphoid tissue and thus more susceptible to infection. Following infection, HPV integrates its DNA into the host cell's genome. This integration disrupts normal cellular functions, primarily through the viral oncogenes E6 and E7 expression. The E6 protein binds to and promotes the degradation of p53, a crucial tumor suppressor protein that regulates the cell cycle and promotes apoptosis in the presence of DNA damage. By inactivating p53, E6 allows for unchecked cellular proliferation and survival. Similarly, the E7 protein interacts with the retinoblastoma protein (pRb), inhibiting its function, which disrupts cellular differentiation and further impedes cell cycle regulation by releasing E2F transcription factors that promote cell division.

The transformation from initial infection to cancer is also influenced by the accumulation of genetic mutations, exacerbated by the continuous expression of E6 and E7 in a backdrop of cellular instability. This viral-induced carcinogenesis modifies not only cell cycle regulation and apoptosis but also impacts the immune response. HPV has mechanisms to evade immune detection, particularly in the oropharyngeal region where immune surveillance is naturally limited compared to more exposed areas like the skin. The progression to oropharyngeal cancer in individuals with persistent HPV infections typically remains clinically silent until malignancy emerges, often leading to diagnoses at more advanced stages. However, despite their advanced stage at diagnosis, HPV-positive oropharyngeal cancers generally respond better to treatments than HPV-negative ones, highlighting the critical role of early detection and preventive strategies such as HPV vaccination in managing the impact of this virus on public health.

Statistical data indicate that in the United States, there were nearly 5,000 more cases of oropharyngeal cancer compared to cervical cancer. Other studies showed that while oropharyngeal cancer had a slow start in the late 1980s in the USA with an upward trend of less than 17%, it gained momentum in the first half of the 2000s and reached a growth of more than 70%. Taking all this into consideration, HPV-positive oropharyngeal cancer can be considered an epidemic.

P16 Overexpression in OPSCC

P16, a cyclin-dependent kinase inhibitor, becomes overexpressed in HPV-positive cancers due to the activity of viral oncogenes. This overexpression serves as a reliable biomarker for the presence of HPV and is associated with improved treatment responses and survival rates. The upregulation of p16^INK4A (p16), a tumor suppressor protein, has emerged as an important biomarker, particularly in the context of OPSCC. This overexpression reflects an intricate cellular response to oncogenic stress, especially induced by high-risk HPV infections, and comprehending its implications provides profound insights into cellular oncogenesis, prognostication, and therapeutic strategies.

P16, encoded by the CDKN2A gene, primarily functions as a cell cycle regulator by inhibiting cyclin-dependent kinase 4 (CDK4) and CDK6, subsequently impeding cell progression from G1 to S phase. It acts as a safeguard, preventing unwarranted cellular proliferation and protecting against potential malignant transformations. Despite its tumor suppressor role, paradoxically, its overexpression is commonly observed in several cancers, predominantly as a response to oncogenic stimuli.

In the context of HPV-related OPSCC, p16 overexpression has emerged as a surrogate marker. The E7 protein of high-risk HPV types (particularly HPV 16) prompts degradation of the retinoblastoma protein (pRb), which is a crucial regulator of cell cycle progression. In retaliation to the loss of pRb function, p16 is overexpressed to mitigate unrestrained cell cycle advancement. Hence, in the context of OPSCC, p16 overexpression is often synonymous with HPV positivity, although exceptions and variances sometimes exist.

The clinical implications of p16 overexpression in OPSCC are significant. It has been corroborated that p16-positive OPSCCs present distinct clinical manifestations and prognoses compared to their p16-negative counterparts. Patients with p16-positive tumors generally have a more favorable prognosis, respond better to treatment (both chemotherapy and radiotherapy), and exhibit enhanced survival rates. This distinction has reformed the management of OPSCC, necessitating reconsideration of de-intensification strategies in treatment approaches, particularly for p16-positive patients, to preserve function and diminish treatment-related complications while maintaining oncological efficacy.

Evaluating p16 status has become integral in diagnostic and therapeutic decision-making processes. Besides aiding in discerning the etiological underpinnings of the tumor, p16 status plays a pivotal role in risk stratification, selection of treatment modalities, and predicting therapeutic outcomes. Notably, with advancements in

targeted therapies and immunotherapies, understanding the molecular profile, including p16 status, becomes vital to select patients who may benefit from such therapeutic interventions.

Special Part

1. A Retrospective Analysis from Western Romania Comparing the Treatment and Survivability of p16-Positive versus p16-Negative Oropharyngeal Cancer

The special part of this thesis includes a comprehensive retrospective analysis comparing treatment outcomes between p16-positive and p16-negative OPSCC patients from Western Romania. Conducted at the Municipal Emergency Clinical Hospital Timisoara, this study involved 60 patients admitted between 2015 and 2019. The patients were divided into two groups: 28 p16-positive and 32 p16-negative. The analysis focused on evaluating their 3-year survivability rates, considering various factors such as age, gender, cancer stage at diagnosis, treatment types, and lifestyle habits like smoking and alcohol consumption.

All patients underwent standardized diagnostic protocols, including ENT examinations, buccopharyngoscopy, endoscopy, biopsies, and imaging studies. The presence of p16 overexpression was confirmed, and treatment protocols primarily consisted of targeted radiotherapy, enhanced with cisplatin chemotherapy. Patients were monitored regularly through follow-ups at 3, 6, 12, 24, and 32 months post-treatment to assess the long-term effectiveness and progression of the disease. Statistical analyses were performed using Fisher's exact test, Chi-square test, and Kaplan-Meier survival analysis.

The study revealed that p16-positive patients had better survival rates and treatment responses compared to p16-negative patients. The average age of p16-positive patients was slightly lower than that of p16-negative patients, aligning with previous studies indicating younger demographics for HPV-positive OPSCC. There was a predominance of male patients in both groups, with a higher prevalence of OPSCC in males, but a significant proportion of females also showed p16 overexpression, suggesting potential HPV implications in the female demographic.

The findings highlight the importance of p16 as a biomarker for HPV in OPSCC, influencing treatment decisions and prognostic assessments. The study also emphasizes the need for public health interventions to address high-risk factors such as smoking and alcohol consumption, which are prevalent in Western Romania. The thesis advocates for the inclusion of p16 testing in clinical practice to improve patient outcomes and optimize treatment strategies for OPSCC.

2. Development and Preliminary Characterization of Polyester-Urethane Microparticles Used in Curcumin Drug Delivery System for Oropharyngeal Cancer

Another significant aspect of this thesis is the exploration of polyester-urethane microparticles for curcumin delivery, aimed at enhancing the therapeutic effects of curcumin in OPSCC treatment. Curcumin, known for its anti-inflammatory and anticancer properties, faces challenges in bioavailability. The study presents preliminary characterization and safety assessments of these microparticles, demonstrating their potential in improving curcumin's bioavailability and efficacy as an anti-inflammatory and anticancer agent.

Curcumin, a natural compound derived from turmeric, has garnered attention for its potential health benefits, including anti-inflammatory and antioxidant properties. Comprised of curcumin, demethoxycurcumin, and bisdemethoxycurcumin—collectively referred to as curcuminoids—this compound has demonstrated inhibitory effects on cell proliferation across various cancer types. Despite its challenges in absorption post-ingestion, curcumin has been the subject of extensive study due to its potential chemopreventive and chemotherapeutic properties, particularly at physiologically attainable concentrations. Its ability to target multiple pathways, known as multitargeted therapy, holds promise for treating a spectrum of diseases, supported by its cost-effectiveness and safety demonstrated in human clinical trials. However, its approval as a therapeutic agent remains on hold, hindered by issues like poor solubility and low bioavailability,

Curcumin interrupts the transformation, proliferation, and invasion of tumor cells. The biochemical pathways linked to cancer development have been thoroughly studied for the past forty years. Over the past twenty years, research has shown that curcumin affects multiple stages of these pathways, highlighting its significant potential in cancer treatment. Epidemiological studies have uncovered a correlation between the low incidence of colon cancers in India and diets rich in starch and curcumin, attributing this phenomenon to their chemopreventive and antioxidant attributes. Furthermore, curcumin's anti-inflammatory properties and its capacity to enhance glutathione production via glutathione-S-transferase activation are noteworthy. Additionally, curcumin's diverse beneficial effects, including wound healing, antiviral, anti-infectious, and anti-amyloidogenic properties, suggest its potential utility in treating Alzheimer's disease.

Curcumin inhibits tumorigenesis through multiple mechanisms. It showcases a blend of anti-inflammatory, antioxidant, immunomodulatory, proapoptotic, and anti-angiogenic properties. Local application of curcumin blocks the formation of DNA-benzo[a]pyrene (B[a]P) adducts in the epidermis. Additionally, it reduces increases in skin inflammation, epidermal DNA synthesis, ornithine decarboxylase (ODC) mRNA levels, ODC activity, hyperplasia, and the formation of c-Fos and c-Jun proteins induced by 12-O-tetradecanoylphorbol-13-acetate. Curcumin also decreases levels of hydrogen peroxide and the oxidized DNA base 5-hydroxymethyl-2′-deoxyuridine (HmdU).

Ingested curcumin blocks benzo[a]pyrene (B[a]P)-induced forestomach carcinogenesis, N-ethyl-N'-nitro-N-nitrosoguanidine inhibiting the appearance of duodenal cancer, and azoxymethane blocking the development of colon cancer. Treatment with green tea alone or combined with curcumin led to reduced cell proliferation in head and neck squamous cell lines. The exposure of these cancer cells to green tea and/or curcumin suppressed the activation of the antiapoptotic transcription factors, activator protein 1 and NF-kB. Curcumin can also reduce the levels of the antiapoptotic proteins Bcl-2 and Bcl-XL, thereby promoting apoptosis through another potential mechanism. Additionally, curcumin downregulates NF-kB, leading to decreased expression of cyclin D1. Consequently, curcumin's growth inhibitory effect is partly due to its suppressive action on the transcription factor NF-kB. NF-kB is a key activator of transcription, and inhibiting this pathway has been shown to suppress tumor growth.

Another target of curcumin is TNF at both the transcriptional and post-transcriptional stages, effectively reducing its expression. Curcumin's suppression of TNF not only curbs the expression of NF-κB but also parallels the effects seen with the neutralization of TNF secretion through an anti-TNF antibody, leading to reduced cell proliferation. Curcumin also impedes the proliferation of cancer cells that are resistant to treatment, downregulating the expression of cyclin D1. Cyclin D1 is essential for regulating the progression of cells through the cell cycle. Furthermore, curcumin initiates apoptosis in cancer cells by stimulating caspase-8. This stimulation leads to the cleavage of Bid, which sequentially triggers the release of mitochondrial cytochrome C and also the activation of caspase-9 and caspase-3.

Curcumin has also provided inhibition to p53 activity. p53 is a key tumor suppressor involved in many cell processes, such as cell cycle control and apoptosis, activating genes that inhibit cancerous growth. However, mutant p53 fails to control cell division, leading to tumor formation. Individuals with one functional p53 gene have a high cancer risk; thus, curcumin could be used as a method of treatment. Through the mechanism of apoptosis, curcumin has a role in regulating the cycle of cell death. There needs to be an equilibrium between the formation and apoptosis of cells, and an imbalanced cycle might provide the set for cancer formation. The primary way curcumin promotes cytotoxicity in tumor cells is by triggering apoptosis. Curcumin reduces the levels of antiapoptotic proteins within the Bcl-2 family while increasing the levels of p53, Bax, and procaspases-3, -8, and -9. Various genes regulated by NF-κB, such as Bcl-2, Bcl-XL, cIAP, survivin, TRAF1, and TRAF2, mainly operate to inhibit the apoptosis pathway. Lastly, curcumin could be used as a drug delivery carrier for medications that currently have limitations because of problems involving solubility, bioavailability, and biodegradability.

In conclusion, this research introduced a novel preparation method designed to address the limitations of traditional polyurethane-based drug delivery systems in oncology, specifically focusing on enhancing their therapeutic efficacy against hepatocellular carcinoma while preserving a strong safety profile on patients with oropharyngeal cancer HPV positive. The study detailed the synthesis and initial characterisation of a synthetic drug delivery system that employs curcumin as the active pharmaceutical ingredient. Polyester-urethane microparticles, ranging in size

from 215 to 271 nm, were engineered, and their release kinetics were studied using a simulated body fluid as a degradation medium.

General Conclusion

This thesis concludes that understanding oropharyngeal cancer's molecular and clinical pathways, particularly concerning HPV and p16, is crucial for developing precise therapeutic strategies and improving patient outcomes. It underscores the necessity of integrating p16 testing into clinical practice. It highlights the promising potential of innovative drug delivery systems, such as curcumin-loaded microparticles, in enhancing treatment efficacy for oropharyngeal cancer patients.

The research emphasises the differences in radiotherapy treatment for HPV-positive patients, who have a more favourable prognosis, respond more quickly to treatment, and, even in advanced stages of the disease, have significantly better chances of recovery compared to HPV-negative patients. Age differences are also significant, with HPV-positive patients being younger, but the most important factor is the radiosensitivity of these tumours. The need to reduce therapeutic doses or even treatment sessions is clear and represents a necessary step towards better management of HPV-positive oropharyngeal cancer patients.

Thus, this research provides detailed insights and practical tools for better patient management and improving the quality of life for HPV-positive oropharyngeal cancer patients.