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PhD THESIS

**MOLECULAR FEATURES OF HEAD AND NECK
CARCINOMAS. DIAGNOSTIC IMPLICATIONS AND
THERAPEUTIC POTENTIAL**

A B S T R A C T

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INTRODUCTION

Due to the increasing incidence of squamous cell carcinomas in the ENT sphere over time, the numerous risk factors involved, and the different age groups affected, a careful study of this pathology is warranted. It is now a major health problem worldwide. Head and neck squamous cell carcinoma (HNSCC) occupies a special place in neoplastic pathology, not only in terms of etiological diagnosis but also in terms of its complex impact on fundamental human functions: phonation and breathing, chewing and swallowing, hearing, psychological and social impact.

As the latest statistical studies show a continuous annual increase in both incidence and mortality, we consider it absolutely necessary to improve primary and secondary prevention, establishing new methods of diagnosis and treatment. Thus, we performed morphological and immunohistochemical (IHC) studies on samples confirmed with HNSCC using the following primary antibodies: E-cadherin, Ki-67, Podoplanin, S100 and CD1a. We obtained in this work important results with a great impact in possible future therapies and demonstrated the role of immunohistochemical markers in disease prognosis, tumor recurrence potential, lymph node metastasis.

We also consider important the early detection of tumors, from the stage of preneoplastic lesions, but also the knowledge of the carcinogenesis process, the elaboration of targeted and personalized treatments for each patient, this being possible by performing IHC analysis with appropriate markers. The importance of studying the molecular peculiarities of HNSCC in a research project is guided by the pursuit of instituting immunohistochemical techniques in current use, putting an accurate diagnosis and implementing a potential modern treatment therapy based on IHC study.

AIM OF THE STUDY

The proposed topic addresses a research project of global importance in the study of squamous cell carcinomas of the head and neck, due to the

aggressive location and progression of the pathology which has a high mortality rate, contrary to various therapeutic approaches.

Over the last few years the formulation of the diagnosis and the application of an appropriate therapeutic approach in HNSCC has become a challenge for ENT specialists with multidisciplinary implications. Statistics have demonstrated an individual, social and economic impact of diagnostic and therapeutic management that requires careful study in terms of the molecular features of squamous cell carcinoma (SCC) as well as the development of effective classification protocols with prognostic and therapeutic value. As recent statistical studies show a continuous annual increase in both incidence and mortality, it is absolutely necessary to improve them, thus increasing the areas of primary and secondary prevention, establishing new methods of diagnosis and treatment. A major emphasis must be placed on early detection of tumours, particularly in high-risk patients, as well as on the knowledge of the carcinogenesis process and the development of modern treatments. The importance of studying the molecular peculiarities of HNSCC in a research project is guided by the pursuit of establishing a correct diagnosis, accuracy and implementation of potential modern treatment therapy.

The aim of this work is the study of molecular peculiarities of HNSCC, the analysis of the most important tumor markers with implication in prognosis, diagnosis and therapeutic potential. The statistics demonstrated an individual, social and economic impact of diagnostic and therapeutic management in HNSCC that requires further immunohistochemical studies in the field.

MATERIAL AND METHOD

The increased sensitivity and specificity of histopathological, histochemical and immunohistochemical methods of analysis, diagnosis and study in the detection of both precancerous and malignant lesions was the starting point in the development of this research study.

Patients and sample processing. Samples were taken from a total of 67 patients (only 50 patients were included in the study) for research purposes at the Department II of Microscopic Morphology, Discipline of Histology of the Victor

Babes University of Medicine and Pharmacy Timisoara (UMFVBT), in collaboration with the Centre for Research in Angiogenesis Timisoara. Prior informed consent was obtained from patients, the principles of the Declaration of Helsinki were followed, and the study was approved by the Scientific Research Ethics Committee of UMFVBT no. 22/September 2019. Formalin-fixed, paraffin-embedded tumor samples were sliced into 4- μ m-thick sections, then they were prepared for hematoxylin and eosin staining, subsequently for immunohistochemical analysis. Samples were histopathologically analyzed and classified according to the Broder system, so the inclusion criterion of patients in the study was histopathological diagnosis of head and neck squamous cell carcinoma (n=50).

Morphological methods. Histopathological diagnosis of HNSCC was established by staining sections with haematoxylin-eosin. Morphological staining was performed using a Leica Autostainer XL (Leica Biosystem Newcastle Ltd, Balliol Business Park West, Benton Lane, New Castle Upon Tyne NE 12 EW, United Kingdom). At the end of the staining, the slides were mounted using the Leica CV Mount (Leica Biosystem Newcastle Ltd, New Castle Upon Tyne NE 12 EW, United Kingdom). The morphologically stained preparations were evaluated and the SCC cases were selected for histochemical and immunohistochemical staining.

Immunohistochemical methods. Selected cases were stained by simple reactions using the following primary antibodies: E-cadherin, Ki-67, Podoplanin, S100 and CD1a. The automated immunohistochemistry machine used was Leica Bond- Max (Leica Biosystems, Newcastle UponTyne, Newcastle UponTyne, UK). Bond Epitope Retrieval Solution 1 and 2, pH 6 and 9 solutions (Leica Biosystems, Newcastle Ltd, Newcastle UponTyne NE 12 8EW, UK) were used for demasking. 3% hydrogen peroxide was used for 5 minutes to block endogenous peroxidase. The next step was incubation with primary antibodies for 30 minutes. The secondary and tertiary antibodies acted for 8 minutes each. Visualization was performed using the Bond Polymer Refine Detection System. It included: Peroxide block (30 ml), 3% hydrogen peroxide; Post Primary Rabbit Anti mouse IgG (10 μ g/mL) in 10% (v/v) buffered sodium triphosphate solution containing animal serum and 0.09% ProClin 950; Polymer (30 ml) Anti-rabbit Poly-HRP-IgG (25 μ g/ml) in 10% buffered sodium triphosphate solution containing animal serum

and 0.09% ProClin 950; DAB Part 1 (2.4 ml) 66 mM 3,3'-diaminobenzidine tetrahydrochloride hydrate, in stabilizing solution; DAB Part B (30 ml), $\leq 0.1\%$ (v/v) hydrogen peroxide in stabilizing solution; DAB Part B (30 ml), $\leq 0.1\%$ (v/v) hydrogen peroxide in stabilizing solution; Hematoxylin (30 ml) $< 0.1\%$. Incubation with DAB chromogen (3,3'-diamino-benzidine) was 10 minutes. Counterstaining was performed with hematoxylin for 5 minutes. This was followed by placing the sections in absolute alcohol for 5 minutes, drying them and clearing them in benzene for another 5 minutes. Mounting was performed in Leica CV Mount automated mode, using a permanent mounting medium such as Entellan.

Quantification methods. Immunohistochemically, cases with cytoplasmic (S100, CD1a, Podoplanin), membrane (E-cadherin) and nuclear (Ki-67) expression in normal structures were included. Examination of sections was performed with the Nikon Eclipse 600 photon microscope (MF). Image analysis and processing was performed using the Axiocam 506 colour microscope, Zeiss, Jena, Germany. Evaluation and digital imaging was performed with the aid of the Panoramic Viewer system (3D Histech, Budapest Hungary).

RESULTS

Cell proliferation is among the most important factors involved in the biological process of ontogenesis. In recent years, significant progress has been made in the development of a number of antibodies associated with proliferation factors. In HNSCC, Ki-67 is the most widely used cell proliferation factor. We performed a study on E-cadherin loss and tumor proliferation rate Ki-67 involvement in HNSCC by immunohistochemical (IHC) methods to evaluate their role as predictive markers of tumor proliferation, aggressiveness and lymph node metastasis. Even though these markers are studied separately, we believe that a study on their correlation in terms of loss of E-cadherin immunohistochemical expression that correlates with a high proliferation rate in HNSCC is needed. The histopathological degree of tumor differentiation should be investigated together with the Ki-67 proliferative index and the ECAD marker to consider the overall results, to draw conclusions about the clinical course and to establish a targeted therapy.

We observed that out of 50 cases, 18% showed loss of ECAD expression, i.e. 82% of primary tumors had high ECAD expression, IRS>10. High ECAD expression was associated with predominant histopathological grade of differentiation G3, this was also due to the high total number of patients diagnosed with grade of differentiation G3 (25 cases), resulting in a significant correlation ($p<0.5$; $p=0.0305$). No association was observed between ECAD expression and patient gender ($p<0.5$; $p=0.6540$), but the increased incidence of males (42 cases) compared to the low number of females who developed HNSCC (8 cases) is consistent with epidemiological risk factors, data and literature studies.

Ki-67 immunoexpression positivity was observed in all 50 samples analysed. The percentage of Ki-67 positive proliferative cells in tumours ranged from 2% to over 75%, with an average of 45-50%. Tumours with less than 10% positive cells were classified as having a low proliferation rate (PR), while tumours with 11-50% and more than 50% positive cells were defined as having a medium and high PR, respectively. 11 tumours (22%) had a low PR, 21 (42%) a medium PR and 18 (36%) a high PR. The majority of G1 differentiation grade tumors had a labeling index <50%, while G2, G3 differentiation grade had a labeling index $\geq 11\%$. There were significant correlations between tumor Ki-67 expression and histological tumor grade ($p=0.0245$), clearly demonstrating the importance of IHC in assessing HNSCC predictive values and tumor prognosis. Cell proliferation index was positively correlated with the degree of cell differentiation and was higher in moderately/poorly differentiated cases, being of real help in establishing a therapy. This finding confirms the idea that the more undifferentiated the SSC is, the poorer the control of the cell division process and the higher the proliferation. This evaluation showed statistical significance between the correlation the of tumoral degree differentiation and the cell proliferation index ($p=0.0245$).

In recent years, cancer treatments have relied on the immune system's ability to recognise and eliminate cancer cells. The ability of dendritic cells (DCs) underpins the generation of anti-tumour immune responses. Thus, we used two important markers, S100 and CD1a protein expression, in the detection of DCs in the studied HNSCC cases. Immunohistochemical evaluation of S100 protein expression revealed the presence of DCs in 42% of cases, with

variable numbers between tumors. Based on DCs analysis, samples were divided into groups according to the evaluation score and correlated with histological grading. The correlation between S100 protein expression by DCs with histological grading was significant ($p < 0.05$, $p = 0.049$). Immunohistochemical evaluation of CD1a revealed positive DCs in 72% of HNSCC cases. The number of positive DCs in tumor tissue increased with the decrease of the histological grade. In addition, the correlation between CD1a expression and histological grade was highly significant ($p < 0.05$, $p = 0.016$). Most of the tumors with G3 differentiation grade had a +1 score for S100 (8 cases) and CD1a (13 cases). Tumors with keratin pearls showed a density of positive DCs for S100 protein and CD1a expression. In addition, the present study found no association between the number of intratumoral and peritumoral DCs.

Over the years, Podoplanin expression has shown a critical role as a prognostic marker in squamous cell cancer. Thus, in the present study we performed a double IHC staining using two important markers, Podoplanin and Ki-67, both used in cancer prognosis. The results show a significant correlation between PDPN immunoexpression and histopathological grading ($p < 0.05$; $p = 0.037$). Mean lymph microvessel density (LMVD) score was calculated using 3 consecutive fields with MFx200 magnification. We found that mean LMVD is higher in the peritumoral area in the G3 classification score (maximum score 13.66x200 MF) than in the intratumoral area (minimum score 0.66x200 MF). We correlated LMVD with the conventional prognostic feature (histological grading of cases), given the possible prognostic implication of LMVD in HNSCC. We did not obtain a statistically significant correlation with tumor grading ($p < 0.05$; $p = 0.577$), but there was a significant correlation with PDPN score ($p < 0.05$; $p = 0.007$). In this study, due to dual Ki-67/PDPN staining, we found 15 tissue samples with lymphovascular invasion (LVI), which were considered on the whole tumor tissue sample. We found no major differences between the peritumoral and intratumoral LVI and there was no significant correlation between LVI and histological grading ($p < 0.05$; $p = 0.976$) or tumor proliferation assessed by Ki-67 expression was not relevant ($p < 0.05$; $p = 0.413$). Immunohistochemical assessment of Ki-67 nuclear staining performed in double staining with PDPN, was observed in all 50 cases. An increased Ki-67 proliferation index was present in 82% of tumor areas, with a statistical

correlation between this and histological grading ($p < 0.05$; $p = 0.050$). In addition, we recorded a statistically significant correlation between Ki-67 expression and PDPN expression ($p < 0.05$; $p = 0.028$).

CONCLUSIONS

Head and neck squamous cell carcinoma (HNSCC) is an important global health problem due to the increasing incidence and the sometimes mutilating impact left on the patient either physically or psycho-socially. We consider this study necessary by addressing and analyzing tumor detection markers with prognostic and therapeutic role of HNSCC. At the same time we highlighted the importance of molecular features of head and neck carcinomas with a role in guided treatment based on tumor profile.

Immunohistochemical expression of E-cadherin (ECAD) and Ki-67 are potential predictive markers of HNSCC. In our study, Ki-67 proliferation index and ECAD expression were found to be important features of HNSCC. Thus, higher tumor proliferation marker Ki-67 correlates with loss of immunohistochemical ECAD expression, indicating poorer patient prognosis. Moreover, this significant correlation is associated with a higher rate of relapse and lymph node metastasis.

The association of dendritic cells (DCs) with histological grading and their intratumoral infiltration suggests their antitumor role, and CD1a immunohistochemical expression, showed strongly significant correlation, represents a useful marker with prognostic and therapeutic potential in head and neck squamous cell carcinoma. On the other hand, this study demonstrated that the expression of dual Ki-67/Podoplanin immunostaining was correlated with the histopathological grade of HNSCC, suggesting that these markers are reliable in clinical use as a prognostic marker of cancer patients. The results of the current study support the importance of dual staining (K-67/Podoplanin) in determining tumor lymphangiogenesis, increasing diagnostic and prognostic accuracy for patients with HNSCC.

We conclude that immunohistochemical markers are important tools that contribute to diagnosis, assess the likely course of disease and predict response

to treatment; thus, they require introduction into routine use in clinical practice. Our study also demonstrated significant impact on the diagnosis and prognosis of HNSCC. Another important feature we demonstrated in this work is the implication of these IHC markers in potential future targeted therapies, and in the personalization of treatment applied to the HNSCC patient.