PROGNOSTIC BIOMARKERS OF HEAD AND NECK CANCER

PhD theses

Ede Birtalan, MD

Semmelweis University
Doctoral School of Clinical Medicine

Supervisor: László Tamás, MD, Ph.D

Official Reviewers: Péter Móricz, MD, Ph.D
Orsolya Dohán, MD, Ph.D

Head of the Final Examination Committee:
Henriette Farkas, MD, D.Sc
Members of the Final Examination Committee:
Tibor Glasz, MD, Ph.D
Zsuzsanna Orosz, MD, Ph.D

Budapest
2018

1. Introduction

Many studies have reported the growing prevalence of human papillomavirus (HPV) in head and neck squamous cell cancers. Chaturvedi et al. observed significantly increased oropharyngeal cancer incidence during 1983 to 2002 predominantly in developed countries and at younger ages; results that underscore a potential role of HPV infection on increasing OPC incidence, particularly among men. Male predominance could be explained by the fact that during active orogenital encounter (assuming heterosexual relationship) males are exposed to a greater HPV load since the infected cervical secretes contain far more HPV virus particles than the infected penis.

Data of large studies show that HPV-positivity rate of oropharyngeal cancers was 24.3% assessed by HPV DNA and $p16^{INK4}$ dual testing. Nevertheless, there was still a lack of consensus concerning the use diagnostic methods needed to detect HPV association. In this study, we assessed the predictive role $p16^{INK4}$-expression versus $p16^{INK4}$/HPV PCR double testing in Hungarian oropharyngeal cancer patients with regard to response to induction chemotherapy.

Immunotherapy has evolved greatly during the last decade and holds the promise of a revolution in the treatment of cancer. There is great enthusiasm towards immunotherapeutic approaches of HNSCC, since it is a disease characterized by profound involvement of the immune system. Checkpoint inhibitors such as anti-PD-1 monoclonal antibody nivolumab and pembrolizumab has recently gained FDA approval for the treatment of recurrent or metastatic HNSCC. Despite intense research, there is still an urging need for prognostic and predictive biomarkers. Besides tumor cell markers, the attention is now focused on markers of immune activation as well.

In this study we investigated the possibly existing differences between subsets of HNSCC based on clinicopathological data and correlations between immunoncological markers. Here, the term subset referred to subgroups according to HPV status and anatomical localization. The

rationale of this approach on one hand was the growing body of evidence that suggests differences in the expression of multiple biomarkers. On the other hand, it became clear in recent years, that HPV associated and non-HPV associated HNSCCs are distinct biologic entities.

2. Objectives

Our first question was weather HPV status is a predictive factor as well. In order to answer this question we compared the response rate of $p16^{INK4}$/ HPV-positive versus $p16^{INK4}$/HPV-negative oropharyngeal cancer patients that were treated with induction chemotherapy.

Our second objective was to investigate the expression of checkpoint inhibitor proteins in HNSCC.

Finally, we also wanted to find out weather checkpoint inhibitor protein expression is related to subsets of head and neck cancer, such as anatomical localization or subgroups based on $p16^{INK4}$/HPV status. Thus, we assessed expression of PD-1, PD-L1, PD-L2 and CTLA-4, just as markers of immune activation: CD8-expression and the rate of tumor infiltrating mononuclear cell infiltration.

3. Methods

Patients

We enrolled 124 therapy naive, consecutively diagnosed individuals with squamous cell carcinoma of the head and neck. We excluded tumors of nasopharyngeal or paranasal sinus localization. Out of this, 110 patients had available tumor blocks for immunohistochemical staining. For the research of immune checkpoint inhibitors we excluded oral cavity cancer patients (N=3) to increase homogeneity and one other patient whose archival tumor block was consumed by previous research, thus did not met the inclusion criteria any more. Doing so we left 106 individuals in the analysis. Each patient underwent treatment between 2012 and 2014 at the Department of Oto-Rhino-Laryngology, Head and Neck Surgery, Semmelweis University (Budapest, Hungary). This research was approved by the Regional, Institutional Scientific and Research Ethics Committee of Semmelweis University (SE TUKEB 105/2014).

Study design

Predictive value of p16^{INK4} and HPV DNA PCR status
First, p16^{INK4} immunohistochemical staining was performed on each tumor sample. Those tested positive for p16^{INK4} underwent subsequent real-time high-risk HPV DNA PCR analysis. P16^{INK4} and HPV DNA PCR double positive samples were regarded as HPV positive. We selected patients who had an oropharyngeal tumor and received induction chemotherapy. Therapeutic response was assessed based on Response Evaluation Criteria in Solid Tumors (RECIST) 1.1. We sought association between p16^{INK4}/ HPV status and therapeutic response (complete remission/partial remission/ stable disease or progressive disease).

Expression of immune checkpoint inhibitors in subsets of HNSCC
We retrieved clinical parameters (localization, stage, grade, gender, smoking habits, alcohol consumption, response to induction chemotherapy, response

to chemoradiotherapy) from our clinical database and utilized information on $p16^{INK4}$/HPV status gained from the previous analysis.
The expression of PD-1 on immune cells and the expression of PD-L1 and CTLA-4 on both tumor and immune cells were observed. We evaluated PD-L2 expression on tumor cells only. The rate of CD8+ mononuclear cells and the proportion of tumor infiltrating lymphocytes (TILs) was assessed as well. We primarily aimed to investigate the prognostic impact of PD-1, PD-L1, PD-L2 and CTLA-4 expression as well as TIL density in HNSCC. In particular we focused on differences between subsets of this disease. Subsets were defined either as subgroups according to anatomical localization or subgroups based on $p16^{INK4}$/HPV status.

Tissue microarray construction

Formalin-fixed, paraffin-embedded (FFPE) tissue block were retrieved from archives of the 2nd Department of Pathology, Semmelweis University. Consequently, all blocks were created using uniform methods based on the local institutional protocol. TMA blocks containing 2 mm diameter cores were created using the TMA Master instrument (3DHISTECH Kft, Budapest, Hungary). TMA blocks contained 50 or 70 cores each. To avoid misrepresentation of samples, 2-3 cores were acquired per tumor. Tissue sections (4 micrometer) were cut on adhesion slides and used for immunohistochemical analysis. Similar sections were cut for DNA extraction and real-time PCR testing.

Immunohistochemistry and evaluation of slides

Immunohistochemistry was performed at the 2nd Department of Pathology, Semmelweis University. After immunostaining, slides were digitalized using a Pannoramic Scan instrument (3DHISTECH, Hungary). Three independent observers blinded to clinical data performed scoring of immunoreactions employing the Pannoramic Viewer software (3DHISTECH, Hungary). In case of inter-observer differences reevaluation took place by all 3 participants and a consensus was reached.

p16^{INK4} staining

BenchMark XT IHC/ISH (Roche, Germany) semi-automated device was used for p16^{INK4} staining with the application of XT UltraView DAB v3 kit. The protocol of staining method was carried out. Briefly, sections were incubated at 72 °C for 4 min. We used EZ Prep Solution (Ventana Medical Systems, Tucson, AZ, USA) three times to remove paraffin. Cell conditioning solution pH 8 (Ventana Medical Systems) was used for heat induced epitope retrieval at 95 °C for 30 min followed by a heating at 100 °C for 4 min. Endogenous peroxidase activity was inhibited with one drop UV INHIBITOR (Ventana), which was applied at 37 °C for 6 min. Primary monoclonal antibody against p16^{INK4} (Clone CINtec E6H4, Ventana) was applied at 37 °C for 32 min in a dilution of 1:100. After incubation with UV HRP UNIV MULT secondary antibody solution (Ventana) at 37 °C for 8 min, peroxidase activity was visualized with diaminobenzidine (DAB) chromogen (Ventana). Nuclear counter-staining was done with hematoxylin II (Ventana). All washing steps were performed with diluted Reaction Buffer Concentrate (Ventana). In each core, nests of at least 200 tumor cells were analyzed by two independent assessors. Cut-off for p16^{INK4}-immunolabelling was set at 75% of cytoplasmic or nuclear staining. Intensity of staining played no role in the evaluation. However, almost all p16^{INK4}-positive samples showed a remarkably strong intensity.

PD-L1 staining

Immunohistochemical staining of PD-L1, PD-1, PD-L2, CTLA-4 and CD-8 was performed similarly. Briefly, all slides were deparaffinized and immunostained with primary antibodies at 42°C for 32 minutes following antigen retrieval. PD-L1 labeling alone required 10 minutes longer antigen retrieval and for PD-L1 the primary antibody incubation time was 2 hours. Detection was performed using a secondary antibody for 60 min at room temperature following the protocols of ultraView™ Universal DAB Detection Kit (Ventana Benchmark Ultra, Ventana Medical Systems Inc., Tucson, Arizona, USA). Positive controls were included in each run showing appropriate results. Slides were counterstained with hematoxylin, washed in water, dehydrated, and cover-slipped before analysis. After initial manual calibration of the optimal dose of the primary antibody, an automatic immunostainer (Ventana Benchmark Ultra, Ventana Medical

Systems Inc., Tucson, Arizona, USA) was used for the serial immunolabeling.

PD-L1 score categories on tumor cells (PD-L1^{TC}) were formed based on the most relevant cut-off values of PD-L1 staining. PD-L1^{TC} categories were: 0: no staining, 1: 0-1%, 2: 1-5%, 3: 6-10%, 4: 11-25%, 5: 26-100%. Scores greater than 1 were considered positive.

PD-L1 staining on immune cells (PD-L1^{IC}) was evaluated as follows: 0: no staining, 1: <1%, 2: 1-5%, 3: >5%. Staining scores higher than 0 were regarded positive.

PD-L2 staining

PD-L2, PD-1, CTLA-4 and CD-8 staining was performed as described above.

PD-L2 immunoscore was allotted to specimens as described elsewhere. The stained tumor cells percentage scores (0: no staining, 1: <10%, 2: 10-50%, 3: >50%) and intensity scores (0: no staining, 1: week, 2: moderate, 3: strong) were summed. The summed score was regarded negative when 0-4 and positive when it was 5 or 6.

PD-1 staining

PD-1 staining on immune cells was assessed: 0: <1%, 1: 1-5%, 2: >5%. Scores 1 and 2 considered to be positive.

CTLA-4 staining

CTLA-4 immunolabeling was investigated on both tumor cells (CTLA-4^{TC}) and immune cells (CTLA-4^{IC}). Given the scarcity of studies describing CTLA-4 expression on tumor cells, we decided to evaluate the samples per 10%; 0: no staining, 1: 1-10%, 2: 11-20%, etc. Positivity was defined when the score exceeded the median value as previously described. The median score of CTLA-4^{TC} was 0 and >1% staining was regarded as positive. The same method was implemented in the assessment of CTLA-4^{IC} staining with >40% immune cell staining regarded as positive.

CD-8 staining
CD8 staining was evaluated as previously described: 0: <1%, 1: 1-5%, 2: 6-20%, 3: >20% High expression was declared if staining of lymphocytes was >20%.

High-risk HPV DNA real-time polymerase chain reaction

Human DNA was extracted from FFPE tissue sections in cases showing $p16^{INK4}$-immunolabelling by QIAmp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) in line with the manufacturer's instructions. High-risk HPV DNA detection was performed using CONFIDENCE™ HPV test (NEUMANN Diagnostics, Hungary) combined with genotyping for HPV 16, 18, 31, 33, 45, 52, 58. The sufficient amount of input DNA was controlled by fluorometric quantitation by Qubit™ Fluorometer (Invitrogen, Carlsbad, CA, USA) using Qubit™ dsDNA HS Assay Kit (Invitrogen) according to the manufacturer's protocol.
CONFIDENCE™ HPV is a TaqMan®-based L1 region specific multiplex real-time PCR assay for viral DNA detection. The test detects HPV 16 and 18 separately and other high-risk types (HPV 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68) in a pooled manner. In case of high-risk HPV presence, genotyping was performed using type specific primers in separated reactions. The quantitative real-time PCR was carried out on QuantStudio™ 6 Flex platform (Thermo Fisher Scientific, Waltham, MA, USA) in 384-well plate format.

Tumor infiltrating lymphocyte (TIL) ratio

The proportion of TIL was assessed on hematoxylin and eosin (HE) stained whole tumor slides by an experienced pathologist (JH). Briefly, TIL scoring was based on the area occupied by mononuclear cells relative to the entire stromal area. Scores were distributed as follows: low: 0-33%, moderate: 34-66%, high: 67-100%.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics for Mac version 20.0.0 (SPSS Inc., Chicago, IL, USA). Pearson Chi-square tests and Fisher exact tests were used to test correlations between discrete variables. In case of survival analysis, Kaplan-Meier estimation with Log Rank test as well as univariate and multivariate regression were applied. All tests were 2-sided and p-values <0.05 were considered statistically significant. The following variables were used in the analysis: gender, tumor localization, HPV status, TNM stage, grade, response to induction chemotherapy, response to chemoradiotherapy, tobacco consumption, alcohol consumption and the biomarkers listed above. Disease-specific survival (DSS) time was calculated using the date of diagnosis and the date of death or last follow-up visit.

4. Results

P16^{INK4}-expression and high risk HPV DNA status

The result of p16^{INK4} immunohistochemistry was available in 110 patients. Out of the 110 tumor samples, 19 cases (17.3%) proved to be p16^{INK4}-positive. The highest proportion of p16^{INK4}-positive cases was observed in oropharyngeal tumors (38.1%), whereas other locations showed much lower (larynx: 4.8%, hypopharynx: 4.2%) or no (oral cavity: 0%) p16^{INK4}-immunolabelling rate.
Out of 19 cases, 9 tumors harboured HPV DNA (HPV-positive cases). HPV 16 was present in 8 cases, HPV 33 in one single case. All HPV-positive samples originated from the oropharynx. This means that the rate of HPV-associated oropharyngeal cancers was 21.4% (9/42 patients). Thus, the specificity of p16^{INK4} to detect oropharyngeal HPV presence was 56.3% (out of the 16 p16^{INK4}-positive oropharyngeal tumors, 9 cases tested positive for HPV as well).

P16^{INK4}/HPV DNA status and response to induction chemotherapy

Of the 110 patients with available immunohistochemical staining, 32 patients received induction chemotherapy. TPF (docetaxel plus cisplatin plus 5-fluorouracil) was given in 30 cases and for 2 patients PF (cisplatin plus 5-fluorouracil) was the choice. P16^{INK4}-positive individuals showed a better response compared with the p16^{INK4}-negative group (Fisher's exact test: $p = 0.025$). There was a significant difference between groups based on HPV status as well (Fisher's exact test: $p = 0.009$).

The impact of TIL rate

Our samples showed a high infiltration rate by TILs. A low TIL score was observed in 27.3% of cases whereas moderate and high infiltration was seen in 21.2% and 51.5%, respectively. Interestingly, PD-L1^{IC} positivity was associated with high TIL rate in the whole patient sample (Chi-square:

p=0.016) and when observed in anatomical subsets separately in the hypopharynx only (Fisher's exact test p=0.006). TIL score correlated positively with CTLA-4^{IC} expression (Chi-square: p=0.013). Observing anatomical subgroups separately, TIL score and CTLA-4^{IC} expression correlated in the hypopharynx only (Fisher's exact test: p=0.028). In oropharyngeal tumors, TIL score was not associated with HPV status (Fisher's exact test: 0.474). We could not find differences in survival nor could we establish association with any other parameters and TIL score.

CD8 expression

High CD8 expression was associated with the presence of PD-L1 on tumor cells (Chi-square: 0.001) and PD-L1^{IC} positivity showed a positive correlation with CD8 status (Chi-square: 0.023) as well. Interestingly none of the samples lacked entirely CD8+ T cell infiltration.

PD-L1 expression on immune cells (PD-L1^{IC})

Considering HPV-negative tumors of all localizations PD-L1^{IC} positivity was proved to be associated with better DSS (HR=0.502; CI95%, 0.273-0.923; p=0.027). In laryngeal tumors, PD-L1^{IC} positivity was associated with improved DSS (HR=0.222; CI95%, 0.062-0.795; p=0.021) compared to the PD-L1^{IC} negative group. In the multivariate analysis controlling for gender, tumor localization and stage, PD-L1^{IC} status did not prove to be an independent prognostic factor. Furthermore, PD-L1^{IC} positivity showed a positive correlation with CTLA-4^{IC} positivity (Chi-square: p=0.049. TIL score correlated positively with PD-L1^{IC} expression as stated above.

PD-L1 expression on tumor cells (PD-L1^{TC}) was not associated with disease-specific survival

PD-L1^{TC} and PD-1 status showed a remarkably strong positive correlation in all patients (Chi-square: p<0.001) and in oropharyngeal and laryngeal localization (Fisher's exact test for oropharynx: p<0.001; larynx: p=0.006), whereas in the hypopharynx the level of significance was not reached

(Fisher's exact test: p=0.074). Negative HPV status was associated with negative PD-L1^{TC} expression in case of oropharyngeal malignancies (Fisher's exact test: p=0.019).

PD-1 expression

Besides the above mentioned data, PD-1 status did not correlate with anatomical subsets (Chi-square: 0.239), HPV status (Chi-square: p=0.601) or with any other parameters observed.

PD-L2 expression

Observing only HPV-negative tumors PD-L2 expression negatively correlated with the presence of PD-1 (Chi-square: p=0.027). None of other biomarkers or clinicopathological parameters were associated with PD-L2 status.

CTLA-4 expression on immune cells (CTLA-4^{IC}) and tumor cells (CTLA-4^{TC})

TIL score correlated positively with CTLA-4^{IC} expression as described above. As mentioned earlier, CTLA-4^{IC} expression correlated with PD-L1^{IC} positivity. We have found that a proportion of HNSCC tumor cells showed faint cytoplasmic positivity for CTLA-4. None of the biomarkers or clinicopathological parameters were associated with CTLA-4^{TC} expression.

Anatomical localization and survival

Laryngeal cancer was characterized by superior DSS when compared to tumors of pharyngeal origin (HR=0.306; CI95%, 0.152-0.616; p=0.001; Log Rank p<0.001). However, this relation became tendential only when observation was limited to patients with locoregionally advanced (stage III-IV B) disease (Log Rank p=0.057).

5. Conclusions

The predictive role of p16^{INK4} immonuhistochemistry versus p16^{INK4}/ PCR double testing regarding induction chemotherapy in oropharyngeal squamous cell carcinoma:

1. P16^{INK4} immunohistochemistry can be considered a possible, precise and widely affordable tool in predictive characterization of oropharyngeal squamous cell cancers in term of response to induction chemotherapy.
2. In comparison with p16^{INK4}/HPV DNA PCR double testing, p16^{INK4} status alone proved to be an equivocally precise indicator of clinical outcome.

Expression of checkpoint inhibitor proteins in subsets of head and neck cancer

3. Our results showed a survival benefit of PD-L1 expression on immune cells in HPV-negative HNSCC.
4. PD-L1^{IC} expression was found to indicate a better prognosis in laryngeal squamous cell carcinoma as well.
5. PD-L2 expression showed no correlation with any parameters observed, except for a negative correlation with PD-1-positive status.
6. We found a proportion of HNSCC expressing CTLA-4 in tumor cell but failed to prove any clinical significance or correlation with other markers.
7. We have not found any remarkable differences between anatomical subgroups of HNSCC.
8. HPV status clearly divided subsets of this disease in terms of cancer immunity.
9. A possibly distinct role of hypopharyngeal localization with regard to immune activity requires further clarification by larger studies.

6. Bibliography of the candidate's publications

Publications related to the thesis

1. **Birtalan E**, Danos K, Gurbi B, Brauswetter, Halasz J, Kalocsane Piurko V, Acs P, Antal B, Mihalyi R, Pato A, Fent Z, Polony G, Timar J, Tamas L. (2017) Expression of PD-L1 on immune cells shows better prognosis in laryngeal, oropharygeal and hypopharyngeal cancer. *Appl Immunohistochem Mol Morphol,* doi:10.1097/PAI.0000000000000590. *(IF:1.634)*
2. Brauswetter D, **Birtalan E**, Danos K, Kocsis A, Krenacs T, Timar J, Mihalyi R, Horcsik D, Polony G, Tamas L, Petak I (2017). $p16^{INK4}$ expression is of prognostic and predictive value in oropharyngeal cancers independent of human papillomavirus status: a Hungarian study. *Eur Arch Otorhinolaryngol,* 274: 1959-1965. *(IF: 1,660)*

Publications not related to the thesis

1. Acs B, Madaras L, Tokes AM, Kovacs AK, Kovacs E, Ozsvari-Vidakovich M, Karaszi A, **Birtalan E**, Dank M, Szasz AM, Kulka J. (2017) PD-1, PD-L1 and CTLA-4 in pregnancy-related - and in early-onset breast cancer: A comparative study. *Breast,* 35: 69-77. *(IF: 2.801)*
2. **Birtalan E**, Dános K, Tamás L. (2016) Immunológiai megfontolások a humán papillomavírus asszociált fej-nyaki daganatok megelőzésében és kezelésében. [Hungarian] *Otorhinolaryngologia Hungarica,* 62: 54–x. *(IF: 0)*
3. Danos K, Brauswetter D, **Birtalan E**, Pato A, Bencsik G, Krenacs T, Petak I, Tamas L. (2016) The Potential Prognostic Value of Connexin 43 Expression in Head and Neck Squamous Cell Carcinomas. *Appl Immunohistochem Mol Morphol,* 24: 476-481. *(IF: 1,634)*

4. Brauswetter D, Danos K, Gurbi B, Felegyhazi EF, **Birtalan E**, Meggyeshazi N, Krenacs T, Tamas L, Petak I. (2016) Copy number gain of PIK3CA and MET is associated with poor prognosis in head and neck squamous cell carcinoma. *Virchows Archiv : an international journal of pathology* 468, 579-587. *(IF: 2,848)*
5. Dános K, **Birtalan E**, Brauswetter D, Tamás L. (2015) A humán (emberi) papillomavírus (HPV) szerepe a szájgarat daganatainak kialakulásában, kórismézében és kezelésében. [Hungarian] - review *Nőgyógyászati Onkológia,* 20,: 8-11. *(IF: 0)*
6. Szentkúti G, Brauswetter D, Dános K, **Birtalan E**, Tamás L. (2014) A fej-nyaki daganatok magas incidenciájának és mortalitásának okai hazánkban. [Hungarian] *Otorhinolaryngologia Hungarica,* 60: 77-80. *(IF: 0)*
7. Kovacs A, Olah A, Lux A, Matyas C, Nemeth BT, Kellermayer D, Ruppert M, Torok M, Szabo L, Meltzer A, Assabiny A, **Birtalan E**, Merkely B, Radovits T. (2015) Strain and strain rate by speckle-tracking echocardiography correlate with pressure-volume loop-derived contractility indices in a rat model of athlete's heart. *American journal of physiology Heart and circulatory physiology,* 308: 743-748. *(IF: 3.348)*
8. Matyas C, Nemeth BT, Olah A, Hidi L, **Birtalan E**, Kellermayer D, Ruppert M, Korkmaz-Icoz S, Kokeny G, Horvath EM, Szabo G, Merkely B, Radovits T. (2015) The soluble guanylate cyclase activator cinaciguat prevents cardiac dysfunction in a rat model of type-1 diabetes mellitus. *Cardiovascular diabetology,* 14:145. *(IF: 4.752)*
9. Oláh A, Németh BT, Mátyás C, Horváth EM, Hidi L, **Birtalan E**, Kellermayer D, Ruppert M, Merkely G, Szabó G, Merkely B, Radovits T. (2014) Cardiac effects of acute exhaustive exercise in a rat model. *International journal of cardiology,* 182: 258-266. *(IF: 6.189)*
10. Radovits T, Olah A, Lux A, Nemeth BT, Hidi L, **Birtalan E**, Kellermayer D, Matyas Cs, Szabo G, Merkely B. (2013) Rat model of exercise-induced cardiac hypertrophy: hemodynamic characterization using left ventricular pressure-volume analysis. *American Journal of Physiology-Heart and Circulatory Physiology,* 305: 124-134. *(IF: 3.348)*