THE EFFECT OF THE MICROENVIRONMENT OF HEAD AND NECK CANCERS ON TUMOR PROGRESSION

PhD theses

Dr. Júlia Lukits

Department of Tumor Progression
National Institute of Oncology, Budapest

Tutor: Prof. Dr. József Tímár

Semmelweis University, Budapest
School of PhD studies

2006

1. Introduction

Head and neck squamous cell carcinomas (HNSCC) show a unique behavior from the point of view of tumor progression. This type of cancer has a clinical course different from most other tumor types, and the metastatic cascade is modified according to the biological behavior of this tumor. Lymphatic dissemination, local reccurrence and neoangiogenesis are the main features of HNSCC progression. Microvessels in most of the tumors are not only a source of oxygen and alimentation but also the key elements of the hematogenous dissemination. In HNSCC, microvessels are mostly a source of oxygen and alimentation and rarely participate in the hematogenous dissemination. Lately, many works focused on the clinical and biological meaning of expression of VEGF (vascular endothelial growth factor) in this type of tumor, especially on the angiogenic type of VEGF. Interestingly, increase of VEGF expression in HNSCC is associated with poor prognosis, independently of microvessel density (MVD), but the mechanisms involved are unclear yet.

The endocrine microenvironment is another important factor in tumor progression in case of cancers characteristically expressing sex hormone receptors as breast (1), prostate (2) or endometrium (3). Sex hormone receptors are also expressed outside the sexual organs, such as in the vascular endothelium (4), the larynx (5) or lung epithelium (6). The presence of the hormone-dependent proteins in normal human laryngeal tissue and laryngeal cancers supports the hypothesis that hormonal mechanisms may play an important role in carcinogenesis (7-9).

Immunotherapy is considered a specific approach to cancer treatment in which activation of immunologic effector mechanisms are used to destroy cancer cells. HNSCC was not considered until recently an immunotherapeutic target, but pioneer studies suggested that immunotherapeutic approaches could well be a new way to manage this otherwise highly aggressive cancer. Until these days head and neck cancer was considered to be an immunosuppressive tumor, contrary to the fact that this tumor type has a rich mononuclear infiltrate whose density is really high in many cases. The clinical significance of immunosuppression in patients with head and neck cancer was demonstrated by the shortened time to recurrence, shortened disease-free survival and shortened overall survival.

2. Aims of the theses

1. Examination of the vascularity of head and neck cancers belonging to different anatomical regions, with different capacity of progression
2. Antiangiogenic effects of radiotherapy in head and neck cancers
3. Molecular identification, expression of estrogen and progesterone receptors in head and neck cancers
4. Stromal reactions after local administration of leukocyte interleukin injections (Multikine) in head and neck cancers

3. Materials and methods

3.1. Patients

A total of 216 patients with head and neck cancer were included in this study. The histological diagnosis in all cases was squamous cell carcinoma. The anatomical localization of these tumors was in the following regions: oral region (tonsilla, oropharynx), 65 cases (30.09%); glottis, 43 cases (19.9%); hypopharynx, 21 cases (9.72%); tongue or base of tongue, 51 cases (23.61%); floor of mouth, 24 cases (11.11%); lip, 12 cases (5.55%). TNM classification was the following: 11 patients had stage T1 (5.16%), 114 stage T2 (53.52%), 65 stage T3 (30.51%) and 23 stage T4 tumors (10.79%). Lymphatic dissemination appeared in 75 patients (35.21%), in 138 patients (64.78%) we could not identify involved lymph nodes, and hematogenous metastasis appeared only in one case. Median age was 62.5 years (range: 41-84 years).

Examination of vascularity was performed on archival tumor samples belonging to Department of Otolaryngology-Head and Neck Surgery of Semmelweis University. The relationship between vascularity and radiotherapy and the expression of hormone receptors in head and neck cancers was examined on surgically resected tumors, from patients treated in the National Institute of Oncology, Budapest. The effects of the local administration of leukocyte interleukin injection (Multikine) was examined on tumors originating from the following clinical sites: Departments of Otolaryngology-Head and Neck Surgery, Dentistry and Oral Surgery of Semmelweis University, Budapest, and Departments of Otolaryngology and Head and Neck Surgery of Uzsoki Hospital, Budapest, Petz Aladár Hospital, Győr, and National Institute of Oncology, Budapest. These examinations were approved by ethics committees of each institution.

3.2. Methods

3.2.1. Immunohistochemistry

Examination of vascularity in head and neck squamous cell carcinomas of different anatomical location were performed on 21 archival tumor samples, using double labeling immunohistochemistry with antibodies against CD31 and laminin. Expression of vascular endothelial growth factor was immunohistochemically examined using anti-VEGF antibody. The effect of radiotherapy on vascularity of oral cancers was examined using paraffin-embedded samples, stained with anti-CD34 antibody. Expression of hormone receptors (estrogen receptor-α ERα, estrogen receptor-β ERβ and progesterone receptor PGR) in head and neck cancers was performed on frozen samples with specific monoclonal antibodies. Examination of the effects of neoadjuvant immunotherapy, using leukocyte interleukin injections, was performed immunohistochemically with antibodies against the following markers: CD68 (macrophages), CD34 (hematopoietic stem cells), CD1a (dendritic cells), anti-myeloperoxidase (neutrophil cells), CD3 (T cells), CD20 (B cells), Ki-67 (proliferating cells), CD25 (interleukin-2 receptor). In all cases, appropriate isotype control antibody was used as negative control.

3.2.2. Histochemistry

The proportion between the quantity of the epithelial component and the stroma in head and neck cancers was determined by two methods: first, connective tissue was stained according to Mallory-trichrom staining, second, slides were labeled immunohistochemically for cytokeratin, using pan-cytokeratin antibody, and then both were morphometrically analyzed.

3.2.3. Morphometry

Evaluation of vascularity of head and neck cancers was performed using Cue-2 image analysis program, at 20x magnification, in five „hot spot" areas, according to international consent (10). Morphometric evaluation of the stromal effects of neoadjuvant immunotherapy was performed using Image Pro Analysis software.

3.2.4. Immunocytochemistry, fluorescent microscopy

Nuclear and cytoplasmic expression of hormone receptors on frozen samples have been viewed by Nikon Eclipse-E600 epifluorescence microscope, equipped with Spot Junior CCD camera and image analysis software.

3.2.5. RT-PCR / nested PCR

Total RNA was isolated from the frozen homogenized tumor samples, possible DNA contamination was eliminated. For reverse transcription random-primer oligo-dT combination was used, the occurrence of reverse transcription was checked by carrying out polimerase chain reaction (PCR) with β-actin primers. For detection of the possible DNA contamination, RNA of the same sample was used as negative control, and DEPC treated water as non-template control. To detect ERα, ERβ and PGR expressions, nested PCR reactions were carried out. PCR products were separated on a 2% agarose gel and detected with Gel Doc 2000 program, after ethidium-bromide staining. Nested PCR products originated from samples of randomly chosen patients were isolated from the agarose gel in the case of all receptor types and all bands, and DNA sequences were determined.

3.2.6. Treatment protocol of the patients treated with neoadjuvant leukocyte interleukin immunotherapy

This study involved two independent examinations. Patients in both examinations, before the administration of leukocyte interleukin injection, were treated uniformly with cyclophosphamide, indomethacin and multivitamin. In the first examination, patients were treated with three different doses of leukocyte interleukin (LI). In patients treated with low and medium doses, 400 IU and 800 IU of LI was administered peritumorally three times a week, through two weeks (2400 IU and 4800 IU final doses, respectively), while patients treated with high dose of LI were given peritumorally 800 IU of LI, five times a week (final dose 8000 IU), also through two weeks. All leukocyte interleukin injections were administered intradermally at the circumferential margin of the visible or palpable tumor mass. In the second examination, LI administration was performed in the following manner: one half of the daily dose (400 IU) was injected perilymphatically and the other half (400 IU) was injected peritumorally over a three weeks' period, five times per week, reaching a cumulative dose of 12000 IU of LI. All LI injections were administered intradermally in the same manner as in the first examination, and the perilymphatic injection was administered at the posterior submandibular area at the jugular lymphatic chain, ipsilateral to the injected tumor. In both examinations, surgery of the tumor mass (or residual tumor mass) was performed

between days 21 and 28 following the initial administration of LI, and locoregional radiation therapy commenced following wound healing.

3.2.7. Statistical analysis

The data were analyzed with chi^2 test, t-test, ANOVA and Kaplan-Meier method.

4. Results and discussion

4.1. Examination of vascularity in tumors of the larynx and hypopharynx

In this study we examined 21 cases in which 11 cases were laryngeal tumors and 10 were hypopharyngeal tumors (I). Histology of the cancers was universally squamous cell carcinoma. The majority of the hypopharyngeal cancers were diagnosed in stage T4, so we presumed that the higher malignancy of hypopharyngeal tumors could be due to the T4 stage and accordingly to the larger size of tumors. Determination of tumor volume of the laryngeal and hypopharyngeal cancers did not support this asssumption because laryngeal tumors had significantly larger size in stage T2 than hypopharyngeal tumors in stage T4. Next, we examined if the smaller size of hypopharyngeal tumors is the consequence of the lower angiogenetic capacity of these tumors. To identify peritumoral microvessels, we applied double labeling immunohistochemistry using antibodies against CD31 and laminin, and microvascular density (MVD) and microvascular perimeter (MVP) were determined by computer-assisted image cytometry. First we compared the MVD of the two types of tumors without respect to the size or stage of these tumors, when results indicated that the MVD is highly similar in the two tumor subtypes (glottic cancers: 211±40 mm^2; hypopharyngeal cancers: 205±20 mm^2). To account for the size differences between these tumors, we compared the MVD of the laryngeal tumors of stage T2 with that of hypopharyngeal ones of stages T4, but the difference was not significant statistically, although the MVD of hypopharyngeal tumors were lower than MVD of laryngeal tumors (178± 20 mm^2, vs. 232± 70 mm^2). There was no difference in the microvascular perimeter (MVP) between the two groups either (larynx cancer: 96±9.9 mm^2; hypopharyngeal cancers: 88.4±7.3mm^2). Morphometric measurements suggest that the angiogenic capacity of the two tumor types is similar. In the case of hypopharyngeal cancers we compared the MVD of the nonmetastatic cases with those producing regional lymph node metastases,

but there were no statistically significant differences. Ultimately, we examined the expression of the most important vascular growth factor in these cancers, with immunohistochemistry using antibody against human VEGF. We stated that in case of these two types of tumors, the frequency of the expression of VEGF-positive tumor cells was identical (larnyngeal cancers: 40%; hypopharyngeal cancers: 41.66%), and the results did not correlate with the stage or size of these tumors. Although the biologic behavior of the cancers of hypopharynx is markedly different from that of cancers of the larynx, the molecular basis of this is not known and has been rarely studied. Frequently, the different invasive/metastatic behavior of tumors is due to a difference in proliferation rate, growth properties, or both. In this study we demonstrated, in a small number of patients, that the angiogenic capacity of the more aggressive hypopharyngeal tumors is the same as in the case of the less invasive laryngeal cancers, and this is why this fact cannot be responsible for the different metastatic behavior. Another result of this study is that the proliferating feature of these tumors cannot be in accordance with the invasive and metastatic characteristics, the invasive and metastatic capacity of hypopharyngeal cancers appearing at a markedly lower size, in comparison with laryngeal tumors. These results lead us to think over the therapy applied for these patients which is effective in the case of laryngeal cancer but poorly effective in the case of slowly growing and highly invasive hypopharyngeal tumors.

4.2. The effects of radiotherapy-induced vascularity changes in oropharyngeal cancers

In this study 35 patients with advanced oropharyngeal cancer were examined (II). Histology of the cancers was universally squamous cell carcinoma. Twenty-one patients were in stage III (60%) and fourteen in stage IV (40%). TNM classification was the following: T1 stage had 4 patients (11,42%), T2 stage- 14 patients (40%), T3 stage– 11 patients (31,42%) and T4 stage had 6 patients (17,14%). In the majority of the cases the tumors were localizated in the tonsillar region and faucial arch (24 cases, 68,57%), then in the base of the tongue (7 cases, 20%), followed by the region of the soft palate (3 cases, 8,57%) and pharyngeal wall (1 case, 2,82%). Eight weeks after completion of radiation therapy, 14 patients (40%) reached complete response (CR), 10 patients (28.6%) had partial response (PR), 4 patients (11.4%) had stable disease (SD), and 7 patients (20%) progressive disease (PD). The median follow-up time was 59 months

(range: 24-67). After five years, 4 (11.5%) of the 35 patients were alive, three of them without evidence of disease, one with a relapse of the primary tumor. The estimated median overall survival was 13 months (95% CI: 8.1-17.9), median progression-free survival was 6 months (95% CI: 4-8). The median MVD score at the beginning of the therapy was 109/mm^2 (SEM: 13.7), which decreased after 20 Gy radiotherapy to 82/mm^2 (SEM: 10.0). Response rate of patients with decreased postirradiation MVD was significantly better (p=0.04) than that of patients whose tumor was characterized by a smaller decrease. Overall survival of patients with low MVD at 20 Gy was significantly better (p=0.012), while progression-free survival showed only a statistical trend (p=0.053).

Tumor growth is critically associated with the vascularization of tumor tissue (11,12). Overgrowth of tumors at advanced stage may result in decreased MVD due to a „dilution effect" but changing angiogenic phenotype can also lead to altered MVD. Therapeutic modalities may also affect the vascularity. Such changes may have prognostic and predictive significance. Some authors did not find any correlation between MVD, response rate and overall survival (13-17). Others have shown that the high MVD is associated with better prognosis and response rate (13,18,19) but some other authors find opposite results (25). Effects on vessels and antiangiogenic properties of radiation have already been investigated in different models (20-23). In our study we analyzed the effect of radiotherapy on MVD. Our findings support the observation of Kourkourakis that HNCC with maintained angiogenic potential during therapy has a poorer prognosis (24). The paramount significance of our study is that our results are the first clinical evidence from a prospective study, supporting the new theory about the significance of anti-endothelial effect of ionizing radiation. Another significant finding of our study is that there is a possibility to detect the response to radiotherapy in an early stage of the treatment of HNCC and that these patients could be selected for a more aggressive treatment. Further studies are necessary to support these options.

4.3. Expression of hormone receptors in head and neck cancers

Sixty-seven HNSCC patients were enrolled in this study, the median age was 64 years (range: 42-86 years), the proportion of men and women was 56/11 (V). Histological diagnosis of all samples was squamous cell carcinoma, and the majority was grade II

(36/67; 53.8%). TNM classification was the following: T1: 1.5% (1/67), T2: 29.8% (20/67); T3: 50.7% (34/67); T4: 17.9% (12/67); predominantly non-metastatic forms (N0M0: 64.2%). In almost half of the cases, localization was in the laryngeal region (32/67; 47.8%), in one third of cases in the oral cavity (24/67; 35.8%), while the others were localized in hypopharynx (11/67; 16.4%). We used two groups, for an easier analysis: oral cavity cancers and laryngeal-hypopharyngeal (LH) tumors. Immunohistochemistry demonstrated frequent nuclear ERα positivity in HNC cells and in normal laryngeal tissue, although cytoplasmic reaction was also observed at lower frequency. ERβ was predominantly found in the cytoplasm of the cancer cells, although nuclear reaction was also detected at lower frequency. PGR protein was found in both the nucleus and the cytoplasm of cancer cells, in the same proportion. Stromal components of HNC did not show sex hormone receptor protein reactivity in any of the cases. Nested PCR analysis of the mRNA extracted from frozen tumor tissues detected authentic ERα and occasionally its δ3 splice variant. ERβ was found to be frequently expressed in two isoforms, wild-type and the δ5 splice variant, while PGR was detected in the authentic wild-type form, all confirmed by sequencing. Furthermore, 2 human HNC cell lines PE/CA-PJ15 and -PJ41, cultured in vitro, were also found to express ERα without the expression of ERβ and PGR, and PJ15 expressed the δ3 variant of ERα as well. About half of HNC cases expressed ER and PGR (28/67 cases, 41.8%). While frequency of ER expression was higher in oral cancers compared to glottic/hypopharyngeal ones (58.3% vs. 46.5%), PGR expression was similar in the two localizations. Solitaire hormone receptor expression was a rare phenomenon in HNC, characterizing a small subset of glottic/hypopharyngeal cancers exclusively (6/43 cases). Since PGR expression is regulated by ER activity, co-expression of ER(s) with PGR is considered a molecular sign of functional ER expression (1). According to this assumption, functional ER expression was a frequent characteristic of HNC, proven to be independent of the anatomical localization (27/67 cases, 40.3%). Neither ER nor PGR expression affected survival of HNC patients: 36-month survival rates were in the range of 55-70%. Furthermore, functional expression of ER in HNC did not affect survival either. Since oral cavity cancer cases were in a minority in this cohort (35.8%), the proportion of receptor-positive and -negative groups of patients were critically unbalanced, therefore we performed a subgroup analysis on laryngeal/hypopharyngeal

cancers. Kaplan-Meier analysis of the data indicated that patients with ER expression (ERα, ERβ or both) in their tumors are characterized by a trend of poorer survival compared to negative cases (p=0.0636). On the other hand, PGR expression status did not affect survival of these patients. Comparison of the ER+ and ER- laryngeal/hypopharyngeal cancers indicated that the T3 and grade 3 cases were over-represented in the ER+ group, while there was no difference in the N stage.

The role of sex hormone receptors in head and neck cancer is controversial. Many research teams focused on the fact that hormone receptors are expressed in „hormone-independent" tissues (26), trying to answer the question why the incidence of head and neck cancer is higher in men, what kind of hormonal mechanisms are playing a role in carcinogenesis and what kind of mechanisms are „protecting" the women from this type of cancer (27). We try to answer the question of the importance of the presence of functional ER in head and neck cancers. This type of cancer is developing mostly in alcoholic men, whose characteristic disease is the alcoholic liver destruction with its complication. Destruction of liver function is followed by the destruction of metabolic processes, naturally including the metabolic processes of sex hormones (estrogen, testosterone) (28,29). Study performed on a large cohort of HNC – alcoholic and nonalcoholic – patients, showed that these patients have higher FSH and LH level and the alcoholic ones have higher estrogen and lower testosterone level (28,29). Another result of this study was that patients with higher FSH and lower testosterone level have a poorer overall survival (28). The survival of laryngeal/hypopharyngeal cancer patients with ER-positive tumors tended to be poorer compared to those with ER-negative tumors, although the difference did not reach the level of statistical significance. These data suggest that ER expression and/or the estrogen-activated ER in this subgroup of HNC may act as a progression promoting factor. However, further studies on a larger cohort of patients is necessary to confirm these preliminary data and to establish if ER plays similar role in oral cavity cancers as in case of laryngeal/hypopharyngeal ones. Our study was done on a relatively small size cohort and the imbalance between the ER+ and ER- tumors did not allow such an analysis. On the other hand, frequent expression of ER, especially together with PGR in HNC suggests that antiestrogens, ER or aromatase inhibitors may have a therapeutic role in the clinical management of HNC.

4.4. The effects of leukocyte interleukin treatment on the microenvironment of oral cancers

In the first study 54 patients participated, 27 were treated with leukocyte interleukin (LI) injection (Multikine) and 27 were the control cases (III). The majority of the tumors were anatomically located in the region of the tongue (22/54, 40.74%), followed by the region of the floor of the mouth (14/54, 25.92%) and base of tongue (9/54, 16.66%), lip (6/54, 11.11%), retromolar region (2/54, 3.70%) and oropharynx (1/54, 1.85%). TNM classification was the following: T1: 2 cases (3.77%), T2: 42 cases (79.24%), T3: 9 cases (16.38%); N0M0: 13 cases (24.52%), N(1-2-3)M0: 40 cases (75.47%).

After leukocyte interleukin treatment we have evaluated the presence and quantity of tumor necrosis and the quantity of proliferating cancer cells, labeled immunohistochemically for Ki-67. The incidence or the absence of any kind of necrosis in OSCC (oral squamous cell carcinoma) was similar in all of the tumors from the different LI-treated groups studied and in the control groups also. Morphometric analysis of the density of Ki-67 positive cancer cells indicated that LI treatment induced significant increase ($p<0.05$) in cycling tumor cells at the lower LI doses administered. On the other hand, the incidence of cycling stromal cells decreased with the increasing LI dose. Mononuclear infiltrates were evaluated in two components of the tumor, in the stromal compartment and in the cancer nests. Using conventional H&E staining, there were no clear-cut differences identified between the tumors resected from the control and LI-treated groups because the control group was also highly heterogeneous in this respect; certain tumors were characterized by a dense leukocytic infiltrate, others by a plasmocytic one and yet others by a lymphoid one. The intraepithelial density of macrophages was comparable to that of the stroma. A relatively high density of intraepithelial macrophages was revealed in the control tumors in a manner similar to ones treated with LI. In this study we were not able to detect CD34+ mononuclear cells.

In the second study (IV) the patients treated with high dose of LI (19 treated and 20 control cases) were similar in the degree of keratinization and histologically. The anatomical localization of the tumors was the following: floor of the mouth: 10 cases (25.64%), tongue: 20 cases (51.28%), lip, oropharynx, bucca, gingiva: 9 cases (23.07%). TNM classification was the following: T1: 1 case (2.70%), T2: 32 cases (86.48%), T3: 4 cases (10.81%); N0M0: 34 cases (91.89%). In two of 19 LI-treated

patients it was not possible to detect any cancer tissue in the surgically resected tumor mass, these patients were considered to be complete responders. In two other LI-treated patients the imaging technique verified more than 50% tumor volume reduction, which was considered partial response. In other four LI-treated patients the volume reduction identified with imaging technique was proven to be more than 30%, which was considered a minor response. Therefore the objective response rate in the LI-treated group was 21%, with an overall response of 42% (8/19 patients). In this study analysis of the effects of LI treatment on OSCC was performed on the LI-treated nonresponder subgroup as well as separately on the responder subgroup. High dose of LI did not change the proportion of cycling cancer cells in responder or nonresponder subgroup. Analysis of macrophage density indicated a down-modulation of the stromal presence of CD68+ macrophages in the LI responder subgroup exclusively and this trend was even more pronounced intraepithelially in the LI-treated group ($p<0.002$ for the LI responder subgroup, $p<0.01$ for nonresponders). In the LI-treated group, increased intratumoral infiltration by neutrophils was observed exclusively in patients with multifocal microscopic necrosis. LI-treated patients exhibited increased neutrophil migration into the cancer nests and neutrophil density was also pronounced in the tumor stroma in the LI responder subgroup. Tumors with high eosinophil density were twice as numerous in the LI-treated group compared with the controls (47% vs. 25%). In the LI-treated group the proportion of the collagenous stroma in tumor tissue was significantly increased compared with the proportion in tumor tissue of control patients ($p<0.05$). Periepithelial collagenosis was similar in frequency in the tumors of both the control and LI-treated patients, whereas interstitial intraepithelial fibrosis was significantly more frequent ($p<0.001$) in the LI-treated group (12 of 17 patients, 70%) compared with the controls (2 of 20 patients, 10%). In the control group, 40% of cases did not show any kind of fibrosis. The degree of stromal fibrosis in the responder and nonresponder subgroup increased in a similar manner in comparison with the control group ($p<0.001$).

The first study (III), where 27 OSCC patients were treated with an immunomodulator investigational drug, leukocyte interleukin, clearly demonstrated that OSCC is an immunogenic tumor and that LI treatment induces lymphocytic infiltration into the tumor. Similarly to other tumor types, the mononuclear infiltrate showed a significant

individual variability in tumors deriving from different patients. This suggests that separate analysis would be necessary to determine which of the components of the cellular infiltrate (T cells, dendritic cells or macrophages) play a significant role in disease prognosis or therapeutic response in OSCC. One of the most interesting findings of this study was that LI treatment induced cell cycle entry of a high proportion of tumor cell population. This effect could well be beneficiary for the patients because one of the major problems facing OSCC radiation therapy or chemotherapy is that both therapeutic modalities primarily affect cycling tumor cells, and those which are not in cell cycle are prone to be more resistant to both chemotherapeutic and radiotherapeutic interventions. In the second study (IV) where high dose of LI was administered to the OSCC patients, important changes were observed regarding the components of mononuclear infiltrate, proportion of tumor stroma and cancer nest and in the objective response to treatment. LI-treated OSCC patients were characterized by a markedly altered pattern of inflammatory cells, suggesting that an acute inflammatory reaction was mediated predominantly by neutrophils and, to a smaller extent by eosinophils and macrophages. Multifocal necrosis of cancer cell nests and an increase in the proportion of connective tissue were detected in the LI-treated patients compared with the controls. These changes may reflect the aftermath of an antitumor immune response raised against OSCC, which was induced by the neoadjuvant administration of LI. Our results indicate that although some degree of antitumor reactivity could be elicited in most patients, this does not necessarily translate into an objective clinical response in all patients. In the case of oral carcinomas treated with LI it is not yet clear which the specific mediators (in LI) are that elicit the antitumor immune response. The increased density of intraepithelial leukocytes in parallel to microscopic necrosis after LI treatment may suggest that the activation of nonspecific antitumor mechanisms is equally important to produce clinicopathologically detectable destruction of the established tumor. These data suggest that the LI-treated group responded homogenously to the treatment but the anticancer effects of LI treatment were heterogeneous. Taken together the findings from both studies, we can conclude that the LI neoadjuvant immunotherapy regimen is a clinically viable approach to the management of OSCC.

5. Conclusions

1. Vascularity is not a critical factor in the progression differences showed by laryngeal and hypopharyngeal cancers.

2. Decrease of microvessel density induced by low dose of radiotherapy shows a significant correlation with better prognosis and response rate in OSCC.

3. About half of head and neck cancer cases expressed ER and PGR (34/67 and 33/67). While the frequency of ER expression was higher in oral cancers compared to glottic/hypopharyngeal ones, PGR expression was very similar in the two localizations. Functional ER expression was a frequent characteristic of HNC. Laryngeal/hypopharyngeal cancer patients with ER expression showed by a trend of poorer prognosis compared to ER-negative cases.

4. OSCCs are pathologically homogenous cancers but are very heterogeneous regarding the density and composition of infiltratig cells.

5. Quantity of cancer cells presenting the Ki-67 antigen increased after low doses of leukocyte interleukin treatment. Interestingly this increase was not detectable in tumors treated with high dose of LI.

6. In patients treated with high dose of LI we obtained objective response in 42.1% of the cases, which was better detectable with pathological examination and quantification of tumor cell nests and tumor stroma proportion as with imaging techniques. We concluded that the tumor stroma reacted similarly after LI treatment in treatment-responder and -nonresponder groups, but the anticancer effects were different, which could be caused by the different sensitivity of the tumors presented at the LI treatment.

6. Publications related to the thesis

I. **Lukits J**, Tímár J, Juhász A, Döme B, Paku S, Répássy G. Progression difference between cancers of the larynx and hypopharynx is not due to tumor size and vascularization. Otolaryngol Head Neck Surg 2001, 125(1): 18-22 **IF: 0.88**

II. Lövey J,* **Lukits J,*** Remenár É, Koronczay K, Kásler M, Németh G, Tímár J. Antiangiogenic effects of radiotherapy but not initial microvessel density predict survival in inoperable oropharyngeal squamous cell carcinoma. Strahlenther Onkol 2006, 182(3): 149-56 **IF: 3.49** (*equal contribution by the two authors)

III. Tímár J, Forster-Horváth C, **Lukits J,** Döme B, Ladányi A, Remenár É, Kásler M, Bencsik B, Répássy G, Szabó G, Velich N, Suba Z, Élő J, Balatoni Z, Bajtai A, Chretien P, Talor E. The effect of leukocyte interleukin injection (Multikine) treatment on the peritumoral and intratumoral subpopulation of mononuclear cells and on tumor epithelia: a possible new approach to augmenting sensitivity to radiation therapy and chemotherapy in oral cancer – a multicenter phase I/II clinical trial. Laryngoscope 2003, 113(12): 2206-17 **IF: 1.449**

IV. Tímár J, Ladányi A, Forster-Horváth C, **Lukits J,** Döme B, Remenár É, Gődény M, Kásler M, Bencsik B, Répássy G, Szabó G, Velich N, Suba Z, Élő J, Balatoni Z, Pocza K, Zemplén B, Chretien P, Talor E. Neoadjuvant immunotherapy of oral squamous cell carcinoma modulates intratumoral CD4/CD8 ratio and tumor microenvironment: a multicenter phase II clinical trial. J Clin Oncol 2005, 23(15): 3421-32 **IF: 9.835**

V. **Lukits J**, Remenár É, Rásó E, Ladányi A, Kásler M, Tímár J. Molecular identification, expression and prognostic role of estrogen and progesterone receptors in head and neck cancer. Int J Oncol (in press) **IF 2,68**

7. Acknowledgements

I would like to express my thanks and gratitude to my supervisor, Professor Dr. Tímár József, who accepted me with confidence in his research team, and created the conditions for my researches and teaching, supported and guided my work through long years.

Thanks to Dr. Kásler Miklós, Head of the National Institute of Oncology, who made it possible to performe my research in his Institute.

Thanks to Professor Dr. Szende Béla and Professor Dr. Kopper László, former and actual Head of the 1st Institute of Pathology and Cancer Research, SE, who provided facilities for my work in their Institute.

Special thanks go to Dr. Rásó Erzsébet, who helped me to get over on all the difficulties appeared in molecular biology, to Dr. Lövey József for working together long time in a good atmosphere, to Dr. Ladányi Andrea for the precious advices given to my dissertation, to Professor Dr. Répássy Gábor, who supported my work through these

years and to Dr. Remenár Éva, for working together in the hormone receptor detection study.

I would like to express my thanks for all the authors of my publications for supporting my work: to Balatoni Zsuzsanna, Bajtai Attila, Bencsik Beáta, Döme Balázs, Chretien Paul, Forster-Horváth Csaba, Gődény Mária, Juhász Attila, Élő János, Koronczay Krisztina, Németh György, Paku Sándor, Pócza Károly, Suba Zsuzsa, Szabó György, Talor Eyal, Velich Norbert, Zemplén Béla.

I am grateful to all members of National Institute of Oncology and the 1st Institute of Pathology and Cancer Research for helping my work.

Finally, I would like to express my thanks to my husband and family, who never gave up trusting me. They supported me continuously and established an optimal background for my studies and scientific work through all these years.

8. References

1. Jensen EV, Jordan C. The estrogen receptor: a model for molecular medicine. Clin Cancer Res 2003; 9: 1890-9

2. Pearce ST, Jordan VC. The biological significance of estrogen receptors alpha and beta in cancer. Crit Rev Oncol Hematol 2004; 50: 3-22

3. Mohler JL, Gregory CW, Ford III OH. The androgen axis in recurrent prostate cancer. Clin Cancer Res 2004; 10: 440-448

4. Venkov CD, Rankin AB, Vaugham DE. Identification of authentic estrogen receptor in cultured endothelial cells. A potential mechanism for steroid hormon regulation of endothelial function. Circulation 1996; 94: 727-33

5. Marsigliante S, Muscella A, Storelli L, Storelli C. Human larynx expresses isoforms of the oestrogen receptor. Cancer Letters 1996; 99: 191-6

6. Omoto Y, Kobayashi Y, Nishida K. Expression, function and clinical implication of the estrogen receptor beta in human lung cancer. Biochem. Biophys Res Commun 2001; 285: 340-347

7. Ferguson BJ, Hudson WR, McCarty KS, Jr. Sex steroid receptor distribution in the human larynx and laryngeal carcinoma. Arch Otolaryngol Head Neck Surg 1987; 113: 1311-5

8. Maiorano E, Botticella MA, Marzullo A, Resta L. Expression of ER-D5 and EGFR in laryngeal carcinoma and malignant epithelium. Acta Otolaryngol Suppl 1997; 527: 95-9

9. Resta L, Marsigliante S, Leo G, Fiorella R, Di Nicola V, Maiorano E. Molecular biopathology of metaplastic, displastic, and neoplastic laryngeal epithelium. Acta Otolaryngol Suppl 1997; 527: 39-42

10. Vermeulen PB, Gasparini C, Fox SB. Second international consensus on the methodology and criteria of evaluation of angiogenesis quantification in solid human tumors. Eur J Cancer 2002; 38: 1564-1579

11. Folkman J. Role of angoigenesis in tumor growth and metastasis. Semin Oncol 2002; 29(6 Suppl 16): 15-18

12. Fox SB. Tumor angiogenesis and prognosis. Histopathology 1997; 3:294-301

13. Gasparini G, Weidner N, Maluta S, Pozza F, Boracchi P, Mezzetti M, Testolin A, Bevilacqua P. Intratumoral microvessel density and p53 protein: correlation with metastasis in head and neck squamous cell carcinoma. Int J Cancer 1993; 55: 739-744

14. Dray TG, Hardin NJ, Sofferman RA. Angoigenesis as a prognostic marker in early head and neck cancer. Ann Otol Rhinol Laryngol 1995; 104: 724-729

15. Gleich LL, Biddinger PW, Duperier FD, Gluckman JL. Tumor angiogenesis as a prognostic indicator in T2-T4 oral cavity squamous cell carcinoma: a clinical-pathologic correlation. Head Neck 1997; 19: 276-280

16. Hegde PU, Brenski AC, Caldarelli DD, Hutchinson J, Panje WR, Wood NB, Leurgans S, Preisler HD, Taylor SG 4th, Caldarelli L, Coon JS. Tumor angiogenesis and p53 mutations: prognosis in head and neck cancer. Arch Otolaryngol Head Neck Surg 1998; 124: 80-85

17. Zatterstrom UK, Brun E, Willen R, Kjellen E, Wennerberg J. Tumor angiogenesis and prognosis in squamous cell carcinoma of the head and neck. Head Neck 1995; 17: 312-318

18. Ravi D, Ramadas K, Mathew BS, Panikkar KR, Nair MK, Pillai MR. Apoptosis, angiogenesis and proliferation: a trifunctional measure of tumour response to radiotherapy for oral cancer. Oral Oncol 2001; 37: 165-171

19. Ito Y, Kamijo T, Yokose T, Kawashima M, Ogino T, Ikeda H, Hayashi R, Sasaki S, Ochiai A. Microvessel density predicts the radiosensitivity of metastatic head and neck squamous cell carcinoma in cervical lymph nodes. Int J Oncol 2001; 19: 1127-1132

20. Kardamakis D, Hadjimichael C, Ginopoulos P, Papaioannou S. Effects of paclitaxel in combination with ionizing radiation on angiogenesis in the chick embryo chorioallantoic membrane. A radiological study. Strahlenther Onkol 2004; 180: 152-156

21. Schultze-Mosgau S, Grabenbauer GG, Wehrhan F, Radespiel-Troger M, Wiltfang J, Sauer R, Rodel F. Histomorphological structural changes of head and neck blood vessels after pre- or postoperative radiotherapy. Strahlenther Onkol 2002; 178: 299-306

22. Schultze-Mosgau S, Rodel F, Keilholz L, Grabenbauer GG, Wiltfang J, Radespiel-Troger M, Sauer R, Neukam FW. Vascularization of free myocutaneous gracilis flaps in replacement transplantation after preoperative radiotherapy. An experimental study. Strahlenther Onkol 2000; 176: 498-505

23. Wehrhan F, Grabenbauer GG, Rodel F, Amann K, Schultze-Mosgau S. Exogenous modulation of TGF-beta(1) influences TGF-betaR-III-associated vascularization during wound healing in irradiated tissue. Strahlenther Onkol 2004; 180: 526-53

24. Kourkourakis MI, Giatromanolaki A, Fountzilas G, Sivridis E, Gatter KC, Harris AL. Angiogenesis, thymidin phosphorylase and resistance of squamous cell head and neck cancer to cytotoxic and radiation therapy. Clin Cancer Res 2000; 6: 381-389

25. Pignataro L, Carboni N, Midolo V, Bertolini F, Buffa R, Cesana BM, Neri A, Viale G, Pruneri G. Clinical relevance of microvessel density in laryngeal squamous cell carcinomas. Int J Cancer 2001; 92: 666-670

26. Kolar Z, Kod'ousek R, Ehrman J Jr, Macak J, Starek I, Kucera J. Expression of estrogen receptors and estrogen-induced proteins in tumors of „hormone non-dependent tissues". Cesk Patol 1994 Feb; 30(1): 12-5

27.) Kleemann D, Kunkel S, Bellin T. Estrogen feedback tests in women with laryngeal carcinomas. Laryngorhinootologie 1996 Feb; 75: 88-90

28. Remenár É, Számel I, Buda B, Orosz Z, Kásler M. „Why men?" Hormones and hormone receptors in male head and neck cancer patients. In: Advances in Laryngology in Europe. Eds: Klainsasser O, Glanz H, Olofsson J, editors. Elsevier, 1997, pp.137-140

29. Yoo HJ, Sepkovic DW, Bradlow HL, Yu GP, Sirilian HV, Schantz SP. Estrogen metabolism as a risk factor for head and neck cancer. Otolaryngol Head Neck Surg 2001; 124: 241-7