Chapter 6

PET with L-[1-¹¹C]-tyrosine in relation to the histopathology of laryngeal carcinomas

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INTRODUCTION

Clinical staging and therapy planning of laryngeal tumors is based on the TNM classification concerning tumor size and extension and metastatic spread to cervical lymph nodes or other organs. It has been shown that tumor stage is the only independent prognostic variable in head and neck tumors, despite an intensive search for other pathological and biological predictive factors 1. However, tumors with similar clinical stage may respond to therapy differently 2, indicating that morphological parameters used in the TNM classification are not sufficient for an accurate estimation of individual treatment outcome and prognosis.

The behavior of disease is suggested to be related to the metabolic activity of tumors. In vitro, the metabolic activity is assessed by histological parameters as mitotic activity, proliferation and differentiation. Although high mitotic rate, high proliferation and loss of differentiation represent aggressive tumor growth in general, these parameters have not been included in the routine staging or therapy planning of laryngeal carcinomas.

In the past years it has been shown that tumor metabolism can be determined in vivo by positron emission tomography (PET), in which radiopharmaceuticals labeled with positron-emitting nuclides are used to assess metabolic and pathophysiological processes. The glucose analogue 2-[^18F]-fluoro-2-deoxy-D-glucose (FDG) is the most widely used radiopharmaceutical in PET and the application of FDG-PET has been successful for a variety of malignancies 3, including primary head and neck cancers and metastatic cervical lymph nodes 4,5. Relations between in vivo glucose consumption as measured by FDG-PET and in vitro tumor grade have been described for different tumor types 6. In head and neck tumors, the relations between metabolic FDG uptake and histopathologic parameters were controversial in several studies 7-9. Furthermore, FDG is not tumor specific, due to accumulation in benign lesions and inflammatory tissues 10.

Amino acids are less avidly metabolized by inflammatory cells and therefore ^11C-labeled amino acids were introduced as radiotracers 11. Uptake of amino acids in tumor tissue is high, due to an increased protein synthesis 12. Methyl-labeled ^11C-methionine (MET) is the most frequently used amino acid, mainly due to the relative ease of synthesis. However, the position of the ^11C-label does not allow for quantitative studies 11. L-[1-^11C]-tyrosine (TYR), a carboxyl-labeled amino acid, is an appropriate compound to determine protein synthesis activity in tumor tissue and by using a dynamic scanning procedure, quantification of the Protein Synthesis Rate (PSR) of tumor tissue is possible 13. TYR-PET has been successfully used in detection and quantification of different primary and recurrent tumors, including head and neck malignancies 14-18.

The aim of the current study was to investigate the presence of relations between in vivo tumor metabolism using TYR-PET and in vitro biological activity of laryngeal carcinomas as reflected by tumor grade, number of mitoses and amount of proliferating cells.
MATERIALS AND METHODS

Patients
TYR-PET was performed in twenty-five patients, 22 men and 3 women, with a histologic diagnosis of squamous cell carcinoma of the larynx (Table 1). Tumors were clinically staged by physical examination of head and neck, endoscopic examination under general anesthesia, biopsies of all suspicious areas of the upper aerodigestive tract and CT imaging according to the TNM classification system (UICC, 1997)\(^\text{19}\). Median age at the time of diagnosis was 64 years (range 46-80 yr). The locations of the twenty-five tumors were 40% glottic, 56% supraglottic and 4% transglottic. The diameter of all tumors was larger than 15 mm.

<table>
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UICC = International Union Against Cancer (1997)
The TYR-PET study was approved by the medical ethics committee of the Groningen University Hospital and written informed consent was obtained from all subjects.

Curative therapy was planned according to the type, location and stage of the disease. Thirteen patients received definitive megavoltage radiotherapy with a conventional fractionation schedule to a total absorbed tumor dose of 66-70 Gy, 2 Gy per fraction, five fractions weekly. Surgery was performed in 12 patients by total laryngectomy, including neck dissection in six subjects. All operated patients received additional radiotherapy to the tumor dose of 60-70 Gy in 30-35 fractions.

**Histological examination**

In all patients, histological diagnosis was made on haematoxilin-eosin stained paraffin sections, with or without additional immunohistological stains, of tumor tissue obtained by diagnostic biopsy during endoscopy. The squamous cell carcinomas were graded according to the UICC grading system (Table 1). Of the twelve patients who underwent total laryngectomy, histological comparison between the larynx specimen and the biopsies was performed to determine possible sampling errors.

The percentage of mitotic figures was counted in ten fields on haematoxilin-eosin stained paraffin sections. Proliferating cells were detected using the monoclonal antibody MIB-1 (DAKO, Glostrup, Denmark), which recognises an epitope of the Ki-67 antigen. Ki-67 is a nuclear antigen present in all parts of the cell cycle except for the G-zero phase of the cell cycle (G0), in which cells are withdrawn from the cell cycle, and the early Gap 1 phase (G1), the phase of the cell cycle before the start of DNA synthesis. Immunohistochemistry was performed on paraffin sections (4 μm) according to a method modified from Shi et al. Briefly, after heating on a hot plate, slides were dewaxed in xylene and rehydrated in serial ethanol washes (100%, 96% and 70%). After one night heating at 80 °C in 0.1 M Tris/HCl buffer solution pH=9.0, peroxidase was blocked using 0.3% H₂O₂. Subsequently, the slides were incubated with the MIB-1 antibody in 1% BSA/PBS (pH=7.4) for 1 hour at room temperature in a humidified chamber. The primary antibody was detected with a rabbit antimouse (DAKO, Glostrup, Denmark) peroxidase labeled secondary antibody diluted in 1:50 + 1% human serum (type AB) followed by incubation with goat antirabbit conjugated peroxidase diluted in 1:50 +1% human serum (type AB). 3,3-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, USA) with imidazole (Merck KgaA, Darmstadt, Germany) in PBS was used as the chromagen according to the manufacturer’s instructions. After counterstaining with Mayer’s haematoxylin, the slides were dehydrated through graded alcohols and mounted with coverslips.

For determining the percentage of mitotic cells and the Ki-67 labelling index (LI), we used ocular micrometry on a microscope by using an eyepiece grid at x400 magnification. Ten fields were randomly selected throughout histologically viable tumor areas. Endothelial cells, inflammatory cells and necrosis were excluded. The number of
mitotic figures and the Ki-67 positive nuclei was then divided by the total number of nuclei in each of the ten fields to calculate the percentage of dividing and proliferating cells per field. The percentage of mitotic cells and the Ki-67 LI was defined as the mean of the indices of the ten fields.

**PET**

Dynamic TYR-PET studies were performed in all twenty-five patients. TYR was produced via a modified microwave induced Bücherer-Strecker synthesis. Synthesis time was 40 minutes, including high performance liquid chromatography (HPLC) purification and testing for sterility, with a radiochemical purity over 99%.

The PET images were acquired using an ECAT 951/31 PET camera (Siemens/CTI, Knoxville, TN). This device has a 56-cm-diameter patient aperture and acquires 31 planes simultaneously. The axial field-of-view is 10.8-cm and the resolution is 6 mm full width of half maximum (FWHM). The head of the patient was fixed in a position with the Frankfurter horizontal plane (line between the external meatus acusticus and the lower orbital rim) making an angle of 110° with the horizontal bed position. Patients fasted for at least 8 hr (except for water and their usual medication) before the study.

A venous canula was placed in the antecubital vein of the forearm for injection of TYR. In the radial artery of the contralateral arm an arterial canula was placed under local anaesthesia. Before injection of TYR, a 20 min transmission scan using a $^{68}$Ge/$^{68}$Ga-source was obtained to correct for photon attenuation by body tissues in the imaged area. TYR was administered intravenously over a 1 min period. The median injected dose was 368 MBq (range 144-399 MBq). Dynamic scanning with 16 time frames was performed from the time of injection to 50 minutes post-injection at the level of the tumor. The protocol included ten 30-seconds images, three 5-minutes images and three 10-minutes images. Simultaneously, arterial blood samples were taken at set time points in order to assess TYR plasma time-activity curve, $^{11}$CO$_2$ concentration and other $^{11}$C-labeled metabolites by radio-HPLC. The plasma tyrosine levels were assessed and ranged from 0.031-0.087 mmol/L, which is within normal values for our laboratory and in accordance with values reported in the literature.

**PET data analysis**

PET data were reconstructed using filtered backprojection by a 0.5 cycle/pixel Hann-filter to obtain transaxial images which were displayed applying standard ECAT software.

To determine tumor PSR, a region of interest (ROI) was placed in the plane with most intense uptake at the site of the tumor as observed at visual analysis, using a 70% threshold of maximum intensity. The tissue time-activity curve obtained from this ROI, together with the plasma-input data (MBq/ml TYR corrected for $^{11}$CO$_2$ and $^{11}$C-labeled proteins) were used to calculate PSR in nanomoles per milliliter tumor tissue per minute (nmol/ml/min) using a modified Patlak analysis. By visually masking nontumor regions.
with physiologically high uptake of TYR (e.g. salivary glands), these regions were prevented from contributing to the average tumor time-activity curve.

Semiquantitative analyses were also performed by calculation of standardized uptake values (SUV) and estimation of tumor-to-nontumor ratio (T/N) to compare absolute quantification (PSR) with semiquantitative methods. The SUV based on bodyweight (SUV_{BW}) was defined as the tumor tissue activity (MBq/ml) in a ROI, as measured by PET, divided by the injected dose (MBq) per kilogram bodyweight. In addition, nontumor uptake (N) was assessed from the right trapezius muscle and the T/N-ratio could be calculated. SUV_{BW} and T/N-ratio were calculated from the summed data obtained of the last three frames (20-50 min postinjection) to reconstruct one static scan.

**Statistical analysis**

To quantify the degree of correlation between PET results and histological parameters, a two-tailed Spearman test (non-parametric parameters) or Pearson test (parametric parameters) correlation analysis was performed. Comparison of the histological grading between the larynx specimen and the biopsy was assessed by a Spearman test. One-way analysis of variance (ANOVA) was used to assess differences between grading groups. A p-value of <0.05 was considered to be significant.

**RESULTS**

In the qualitative evaluation of TYR-PET studies, all 25 primary malignancies were identified correctly (Fig. 1). The tumor PSR ranged from 0.87 to 6.96 nmol/ml/min (median 2.32 nmol/ml/min). The median SUV_{BW} and T/N-ratio of tumor tissue were 4.62 (range 1.85-7.34) and 4.85 (range 2.33-9.09), respectively. In one patient, only SUV and T/N-ratio could be calculated, due to the absence of arterial cannulation for technical reasons.

**Figure 1.**

*TYR-PET scan of a patient with a T2 N0 supraglottic laryngeal squamous cell carcinoma.*

UICC grading showed 9 well differentiated (G1), 10 moderately differentiated (G2), and 6 poorly differentiated (G3) tumors (Table 1). Glottic laryngeal carcinomas contained mainly high differentiated tumors (56% G1), whereas 76% of the supraglottic tumors were moderately (G2) or poorly (G3) differentiated. The mean PSR values for G1, G2 and G3 tumors were 1.91 nmol/ml/min (range 1.14-3.75), 2.60 nmol/ml/min (range 0.87-6.96) and 3.49 nmol/ml/min (1.62-5.12), respectively (Fig. 2a). Statistical analysis (one-way ANOVA) could not demonstrate significant differences in PSR values (p=0.083) between G1, G2 or
Tumor grade (G1 = well differentiated; G2 = moderately differentiated and G3 = poorly differentiated) versus protein synthesis rate (PSR) (Figure 2a), standardized uptake values (SUV_{BW}) (Figure 2b) and tumor-to-nontumor ratio (T/N) (Figure 2c) as measured by PET of laryngeal and hypopharyngeal carcinomas. Mean values are reflected as horizontal lines in each grading group. A tendency (p=0.083) was observed between PSR values and grade.

Figure 2.

G3 tumors. Differences in SUV and T/N-ratio between the different grading groups were not significant either (Fig. 2b and 2c). When supraglottic tumors were evaluated separately, the mean PSR values for G1, G2 and G3 tumors were 1.55 nmol/ml/min (range 1.23-2.32), 2.60 nmol/ml/min (range 2.40-2.81) and 4.69 nmol/ml/min (4.16-5.12), respectively (Fig. 3). Although statistical analysis was not valid because of the limited number of patients, an association exists between increase of metabolic activity and loss of differentiation in tumors.

Mitotic activity ranged from 0.06-4.3% with a median value of 0.56%. No correlations were observed between the mitotic activity and PSR, SUV_{BW}, or T/N-ratio as measured by PET (Fig. 4).
Figure 3. 
Protein synthesis rate (PSR) as measured by TYR-PET versus tumor grade of supraglottic squamous cell carcinomas.

Figure 4. 
In vitro mitotic activity versus in vivo protein synthesis rate (PSR) as measured by TYR-PET. No significant correlation (p=ns) could be found.

Darkly stained positive nuclei were clearly distinguished from negative nuclei (Fig. 5). Ki-67 LI varied from 8% to 69% with a median percentage of 24%. No correlation was observed between the percentage of cells in proliferation and PSR, $\text{SUV}_{\text{BW}}$ or T/N-ratio (Fig. 6).

Figure 5. 
Monoclonal antibody MIB-1 stained tissue sections of a supraglottic tumor (20x; figure 5a, and 200x; figure 5b, respectively) showing a low amount of proliferating cells. All dark nuclei express Ki-67 and have entered the cell cycle.
DISCUSSION

The current study was set up to assess a relation between in vivo metabolic activity of laryngeal squamous cell carcinomas as measured by TYR-PET and in vitro biological parameters as determined by histopathological techniques.

The growth rate of tumors, and therefore metabolic activity, is an essential feature in their malignant potential. In histopathology, the malignancy grade is associated with the aggressiveness of growth. Although loss of differentiation in tumors results in a less efficient and therefore higher use of energy, and higher mitotic rate and proliferation activity require more energy, a clear correlation between histologic parameters and tumor metabolism has not been demonstrated.

![Graph showing Protein synthesis rate (PSR) versus proliferation activity.](image)

**Figure 6.**
Protein synthesis rate (PSR) as measured by TYR-PET versus proliferation activity. The correlation between PSR and the Ki-67 labeling index (LI) was not significant.

**PET versus histological grade**

In FDG studies, a relation between histological grade and glucose uptake has been controversial. In patients with soft-tissue sarcomas 22, non-Hodgkin lymphomas 23 and brain tumors 24 FDG uptake was related to histological grading. However, a discrepancy between FDG uptake and grading was described in patients with head and neck cancer. In a FDG study on thirty-seven patients with different head and neck tumors, Minn et al. described a strong association between high FDG uptake and low or moderate histological grade 9. However, in several other studies on head and neck tumors, no clear correlations were found 8,10,25. Correlations between histological grade and MET uptake were found in brain, lung and uterine cancer 26, but not in patients with head and neck carcinomas 27.

TYR-PET studies for comparison with histological grading have been performed in brain and soft tissue sarcomas. Brain tumors demonstrated no correlations between histological grading and TYR uptake 15,28. In soft-tissue sarcomas, Kole et al. described a moderate correlation (r=0.58) between PSR and grade 29, while Plaat et al. could not confirm this result 30.
In the present study with a small number of patients, the quantitative metabolic values did not differ significantly between the tumor grades, although a tendency (p=0.083) appears to be present. The differences were even more evident when supraglottic tumors were evaluated separately, suggesting a relationship between in vivo metabolic activity and tumor aggressiveness. Distinction between glottic and supraglottic tumors was made because of heterogeneity of laryngeal carcinomas. Although there is no overwhelming evidence to support the view that tumors from different sites of the larynx have intrinsic biological differences, supraglottic tumors do tend to be more moderately and poorly differentiated than glottic tumors. This is in accordance with the results of our study.

**PET versus proliferation and mitosis**

High proliferative activity in tumors indicates that a high proportion of tumor cells has entered the cell division cycle (G1, S and G2 phases), whereas a high mitotic rate implies that a large number of cells is in the final phase of the cell cycle (M phase). Proliferation and mitosis are markers of the biological behavior of malignant tumors. In patients with intracranial tumors and bronchial carcinomas, FDG uptake was shown to be related to proliferative activity. In head and neck tumors the results were not conclusive. Initial studies by Minn et al. revealed a relation between glucose metabolism and proliferation of head and neck tumors based on DNA flow cytometry data. They suggested that the increase in glucose utilization in head and neck tumors is mainly needed for nucleic acid synthesis. However, in a FDG and flow cytometry study by Haberkorn et al., no linear correlation between FDG uptake and proliferation index was found. Only when the head and neck tumors were separated in groups with high and low FDG uptake, correlations could be demonstrated. These data suggest that other factors contribute to the uptake of FDG in tumors. Also in vitro studies using FDG-PET produced contradictive results. Higashi et al. found that FDG uptake did not correlate with proliferative activity, but strongly related to the number of viable tumor cells. A recent study using Ki-67 labeling demonstrated a correlation with FDG uptake and proliferation kinetics in head and neck tumors, which is in contrast to previously reported results.

The outcome from studies comparing FDG and MET uptake with histopathological parameters are not conclusive either. Whereas Minn et al. found that MET uptake represented proliferative activity and FDG was a marker of cell viability, the opposite conclusion was reached by Kubota et al.

Studies comparing mitotic and proliferative activity with TYR uptake have been performed in brain and soft tissue sarcomas. In two studies of patients with various brain tumors, no correlation was found between proliferation and PSR, as measured with TYR-PET. Two studies on soft tissue sarcomas demonstrated correlations between PSR and proliferation, but in only one study a relationship was observed with mitotic rate. In the present study on patients with laryngeal carcinomas, no correlations were observed between mitotic rate or proliferative activity as measured by Ki-67 labeling and quantitative TYR-PET values.
Several different explanations should be considered for the inconsistency in observations of the different TYR-PET studies. Due to a restricted research setting of PET studies and the relatively low incidence of head and neck tumors, the number of patients is necessarily limited and heterogeneous. Furthermore, neoplasms differ in heterogeneity on the cellular level, and may also be macroscopically heterogeneous, due to differences in distribution of cell populations, vascular structures, stroma and necrotic tissue. Schmidt et al. have demonstrated that tissue heterogeneity can have significant effects on the quantitative parameter values \(^{36}\). The differences in TYR-PET observations between brain tumors, soft tissue sarcomas and head and neck carcinomas may be caused by inter- and intratumor heterogeneity.

Also, in analyzing the relationship between tracer uptake and histological parameters, the intratumor heterogeneity of squamous cell carcinomas of the larynx may cause sampling errors in the histological evaluation. The TYR uptake, based on a standardized ROI analysis, encompasses the entire tumor, whereas histological parameters are evaluated on a representative tissue sample. Sampling errors were reduced by analyzing randomly selected microscopic areas within the tumor sample. Although we found relatively high correlations \((r=0.87)\) between grading of the samples of the larynx specimen and the biopsies in the present study, sampling errors may still have occurred and have influenced the relations between histological parameters and PET results.

Cell proliferation has been demonstrated to increase in almost all tumors and is often measured by immunostaining the Ki-67 protein. However, several reports demonstrated that Ki-67 does not have prognostic value in predicting therapy outcome of head and neck tumors \(^{31}\). The limited number of patients and the heterogeneity of the tumors was often mentioned as explanation for these results, but it can also be argued that Ki-67 is not the optimal parameter for assessment of tumor growth of head and neck tumors. Furthermore, proliferative activity does not seem to have a significant relationship to histopathological grading in head and neck tumors \(^{37}\). This could explain the fact that in the present study a relation was observed between TYR uptake and grade, but not between TYR uptake and proliferative activity. In vivo metabolic activity of tumors has been suggested to be related to the behavior of the disease, and in head and neck tumors, FDG uptake was demonstrated to be a prognostic factor \(^{9}\). Our results suggest that in vivo metabolic activity using TYR-PET reflects tumor aggressiveness since an association was observed between TYR uptake and tumor grade, but not with proliferation or mitotic activity of laryngeal squamous cell carcinomas. Further investigation of histopathological and biological parameters in larger series of patients with laryngeal carcinomas is essential for assessment of future applications of TYR-PET. On the other hand, the development of future histopathological tumormarkers may reveal other relationships between histopathology and PET.
CONCLUSIONS

In the present study the relationships between in vivo tumor metabolism using TYR-PET and in vitro biological activity as reflected by tumor grade, mitotic and proliferative activity of laryngeal squamous cell carcinomas, were investigated. An association was found between TYR uptake and tumor grade, but not with the number of mitoses or the amount of proliferating cells measured by Ki-67 immunostaining. These results may suggest a relation between in vivo TYR uptake and aggressiveness of tumor growth.
REFERENCES


