Characterization of a New Rat Model of Head and Neck Squamous Cell Carcinoma

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Abstract. Aim: To develop and characterize by imaging and pathological examination a new immunocompetent rat model of head and neck squamous cell carcinoma (HNSCC). Study design: Prospective animal research. Materials and Methods: Frozen specimens of HNSCC induced chemically by 4-nitroquinoline 1 oxide (4-NQO) in Sprague Dawley rats were used for the first graft. Serial allografts were then performed with fresh specimens of tumor in twenty-five Sprague Dawley rats. A specimen of tumor (100 mm³) was picked up by head and neck dissection during an autopsy. The graft was performed in a subcutaneous manner, in the ventral part of the neck, using an incision of 4 mm, through the masseter muscle. Tumors were clinically measured once a week and volumes were calculated. 2-[¹⁸F] Fluoro-2-deoxy-D-glucose positron emission tomography coupled with computed tomography (FDG-PET/CT) was performed on days 14 and 30 after the graft. Rats were euthanized and pathological features were assessed using hematoxylin-eosin (HE) staining and immunohistochemistry markers to characterize the tumor. Results: An 80% take rate was achieved using fresh tumor specimens. Tumors grew rapidly; the mean tumoral volume was 1.013 cm³ on day 14 and 7.994 cm³ on day 30. FDG-PET/CT imaging targeted regions of metabolically active tumor. It showed a uniform uptake of ¹⁸F-FDG on day 14 and a large area of central necrosis on day 30. Pathological examinations showed a typical squamous cell carcinoma, with similar immunohistochemical analyses to the human squamous cell carcinoma. Conclusion: We propose a new allograft HNSCC rat model which is easily reproducible and rapidly obtained in comparison to that induced chemically with 4-NQO. This model was developed in immunocompetent rats, with similar conditions to human carcinogenesis and could be used for testing new therapeutics.

Squamous cell carcinoma (SCC) is the most common kind of head and neck neoplasms. To perform animal studies, a representative and reproducible tumor model in animals is needed. The chemical rodent model using 4-nitroquinoline 1 oxide (4-NQO) used in application or in drinking water is well-known and described in various studies (1-6). It allows various localisations of HNSCC to be obtained but requires a considerable waiting period for preparation, at least 34 weeks. Other models are described in the literature, using xenograft of human SCC or allograft of SCC in nude rats (7, 8). For us, they are not realistic models for testing therapeutics because the conditions are not the same as in human clinical practice. We report the setup and characterization of a new HNSCC model established by allograft in immunocompetent rats.

Materials and Methods

Animal models and experimental protocol. All procedures and care given to the rats were performed according to institutional guidelines. Twenty-five immunocompetent male rats (Sprague Dawley), 21 days old and weighing 30 g were used for the study. They were kept in plastic cages, with two animals in each cage, and were fed with standard rat diet and drank water ad libitum. A day-night cycle of 12:12 hours was maintained at a temperature of 20°C. During each animal handling procedure including grafting, radiotracer injection and animal imaging, the animals were anesthetized with 1% to 3% isoflurane (Baxter, Belgium) in 100% oxygen, using an animal anesthetizing evaporator (Minerve, Esternay, France).

The first graft on five rats was made with tumor chemically induced by 4-NQO. This solution was administrated to two rats in drinking water ad libitum during 34 weeks. The 4-NQO solution was prepared weekly from a stock solution (1 mg/5 l), which was diluted with tap water to obtain a concentration of 0.001% for the experiment. The bottles were refilled once a week with fresh 4-NQO solution. All rats presented an HNSCC, 34 weeks after the start of the experimentation. Tumors essentially located on the base of tongue were removed during the autopsy, cut into specimens and frozen at –80°C in a solution of foetal bovin serum (50%) (Gibco),

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RPMI (40%) (Gibco) and DMSO (10%) (dimethyl sulfoxide; Sigma) to be preserved. Before the graft, specimens of tumor were defrosted for few minutes in calf foetal serum, than cut into sections of 100 mm³.

In the first group, five rats were grafted subcutaneously, through the masseter muscle both on the right and left sides of the ventral part of the neck, using an incision of 4 mm. The incisions were closed with 4.0 silk sutures. Tumors obtained in this first group were removed at day 14 and grafted directly with fresh specimens into the second group established by twenty rats, using the same protocol. All the rats were examined on a daily basis to follow the tumor progression. The growth curves from ten rats of the second group were reported. Tumor dimensions were evaluated by callipers’ measuring the diameters (a: longest diameter; b: shortest diameter) of each tumor. Tumor volumes were calculated according to the formula established by Carlsson: \( V = a \times b^2 \times \frac{1}{2} \) (9).

**FDG-PET/CT.** FDG-PET/CT was performed for ten rats of the second group at days 14 and 30 after the graft. All of these rats had clinical neck masses on both sides of the ventral neck. Food was withheld for 12 hours and 2 hours before the imaging 18.5 mBq/100 g of \(^{18}\text{F}-\text{FDG}\) (Cyclopharma, Laboratoire Saint Beauzire, France) were injected into a tail vein. Small animal imaging studies were performed with a combined PET/CT (Biograph 6) approved for human clinical use (Siemens Medical Solutions, Knoxville, TE, USA). This CT allowed simultaneous acquisition of 325 transaxial slices of 0.6 mm, for each bed position of PET acquisition. Transaxial PET resolution was 4.5-mm full width at half maximum (FWHM). The technical parameters used for the CT portion of PET/CT were a detector row configuration of 6x0.5 mm, pitch of 1.8 mm, voltage of 80 kVp and a current of 130 mA. This acquisition mode was used for attenuation correction and automatic image fusion system. The field of view (FOV) chosen for CT reconstruction was 25 cm. CT data were reconstructed by classical filtered back projection to obtain transversal slices of 1 mm thickness.

A 10 min 3D acquisition of one bed centred from the nose to the abdomen was performed after the CT. PET data were reconstructed after an iterative process (8 iterations, 16 subsets) with post filtration by using a low pass 1.0 threshold to obtain 337x337 pixels image size, zoomed by a factor 2. Contiguous 2.0 mm transaxial, 2.7 mm sagittal and coronal slices were obtained. The tumoral uptake of \(^{18}\text{F}-\text{FDG}\) was quantified in SUVs (standardized uptake value). SUV is the tumor

**Table I.** FDG-PET/CT results at days 14 and 30 after the allograft (n=10). Values are means±standard deviation.

<table>
<thead>
<tr>
<th>Days after allograft</th>
<th>14</th>
<th>30</th>
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</thead>
<tbody>
<tr>
<td>Global tumoral volume (cm³)</td>
<td>1.265±0.289</td>
<td>8.2675±5.407</td>
</tr>
<tr>
<td>Metabolic tumoral volume (cm³)</td>
<td>1.265±0.289</td>
<td>1.9625±0.758</td>
</tr>
<tr>
<td>Necrosis volume (cm³)</td>
<td>0</td>
<td>6.305±4.771</td>
</tr>
<tr>
<td>Mean SUVmax</td>
<td>2.945±1.237</td>
<td>2.5775±0.655</td>
</tr>
<tr>
<td>Mean SUVmean</td>
<td>1.895±0.53</td>
<td>1.475±0.221</td>
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</tbody>
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**Figure 1.** Sprague Dawley rat. Presence of a tumor located on the ventral part of the neck at day 30 after the allograft.

**Figure 2.** Tumor growth curve at days 7, 14, 21 and 30 after the allograft (n=10).
activity concentration (MBq/ml) normalized by the injected dose (MBq) per body weight (ml). Both SUV max and SUV mean were determined. Tumoral metabolic volume was calculated using 40% max threshold VOI (volume of interest).

**Pathology.** One day following the second FDG-PET/CT, rats were euthanized under isoflurane sedation. Tumors, lungs and liver were dissected and fixed with 10% buffered formalin. Each sample was sectioned and embedded in paraffin. Five-μm-thick sections of each

*Figure 3. FDG-PET/CT at day 14 after the allograft. CT in grey scale – PET/CT coregistered with grey scale for CT and hot metal for PET in coronal slices: $^{18}$F-FDG uptake on the right tumor (red arrow) and the left tumor (white arrow) developed on the ventral part of the neck.*

*Figure 4. FDG-PET/CT at day 30 after the allograft. CT in grey scale – PET/CT coregistered with grey scale for CT and hot metal for PET in coronal slices: $^{18}$F-FDG activity predominated at the rim of tumors (red arrows) with a notable decrease of fixation from the tumor’s surface to its necrotic center (white arrow).*
specimen were prepared, stained with hematoxylin-eosin (HE) and microscopically analysed. Immunohistochemical staining was carried out using monoclonal mouse anti-human cytokeratin, clone AE1/AE3 and clone 5/6 in order to characterize the tumor.

Results

We observed development of a mass, on one side of the neck for 3 rats (30% take rate) of the first group and on both sides for 16 rats of the second group (80% take rate) (Figure 1). Mean volumes were calculated on days 7, 14, 21 and 30 after the graft for ten rats of the second group (Figure 2). In the second group, tumors on both sides progressed independently with similar volumes on day 14 (standard deviation: 0.3 cm$^3$) and a high disparity of volumes on day 30 (standard deviation: 5.9 cm$^3$).

FDG-PET/CT results. On day 14, the FDG-PET/CT confirmed the presence of tumors clinically diagnosed by a local accumulation of $^{18}$F-FDG. It showed the extension of tumors and determined the evolution of metabolic tumoral volumes between days 14 and 30. The intratumoral distribution of $^{18}$F-FDG was uniform on day 14 when the mean tumoral volume was $1.265\pm0.289$ cm$^3$ (Figure 3). On day 30, the mean tumoral volume was $8.267\pm5.407$ cm$^3$ and a central hypofixation of $^{18}$F-FDG was noted for all tumors (Figure 4). $^{18}$F-FDG activity predominated at the medial rim of tumors with a notable decrease of fixation from the tumor’s surface to its center. Global volumes and metabolic volumes were measured and necrosis volumes were calculated (Table I). Means of SUVmax and SUVmean were determined. No pathological uptake was found in liver or lungs.

Pathological results. The HE-stained sections of the allograft tumor showed lobulated nodular sheets and islands of SCC. The tumors displayed a circumscribed broad pushing growth front with a fibrovascular capsule and intervening fibrous septae. The tumor cells consisted of pleomorphic squamous epithelial cells exhibiting round to oval vesicular nuclei with prominent nucleoli, pink cytoplasm, scant keratinisation and atypical mitotic figures (Figure 5). The tumors exhibited confluent geographic areas of central necrosis with small residual nests of viable cells at the periphery. The specimens examined using immunohistochemical stains were found to have a positive expression with cytokeratin AE1/AE3 and a strong positive expression with cytokeratin 5/6 that confirmed the squamous nature of the carcinoma (Figure 6). Microscopic examination of HE-stained sections of lungs and liver revealed no evidence of tumor metastasis.
Discussion

This is a new HNSCC rat model which presents lots of advantages, in comparison with that with HNSCC induced with 4-NQO. Tumor growth can be quickly obtained, in less than 7 days after the graft. Clinical examination and the following of tumor growth are facilitated by the subcutaneous positioning of the graft. By using this graft protocol, we propose a reproducible model of HNSCC in order to obtain a uniform cohort.

It is an immunocompetent experimental model, which is more realistic than that using xenografts or the subcutaneous inoculation of human SCC cells, or allograft models obtained on nude rats. We propose an HNSCC with similar pathological and immunohistochemical characteristics to human HNSCC. In our experiment, we observed that the graft take rate was much higher with fresh tumor than with frozen specimens. We noted the importance of selecting the tumoral fragment used for the graft, in the peripheral area, in order to increase the take rate. FDG-PET/CT was useful to point the metabolic regions of the tumor often located on the median peripheral area. The graft take rate is also higher when the removal of the tumor is performed on day 14 because there is less necrosis. In our experimentation, we opted for grafting on both side of the neck in order to increase the take rate. The age of the rats at graft time was also important. We chose rats aged 21 days which is the weaning rat age, when the risk of graft rejection was reduced. Other experiments in our research unity showed that tumoral graft was always rejected when it was made in fully grown immunocompetent rats. This experimentation confirms that the tumor could be maintained by serial transplantation in young immunocompetent Sprague Dawley rats.

PET imaging with FDG is used routinely in clinical oncology. 18F-FDG is a glucose analog that accumulates in metabolically active tumors. Our experiment confirms that FDG-PET/CT can be used for HNSCC detection and measurement of metabolic tumoral volume. Metabolic imaging confirmed the presence of tumors diagnosed by clinical examination and showed tumoral extension. We observed a good correlation between clinical measurement of tumoral volumes and global tumoral volumes evaluated by FDG-PET/CT at days 14 and 30. FDG-PET/CT at day 14 showed a uniform intratumoral distribution of 18F-FDG, correlated with an absence of necrosis, while at day 30, a heterogeneous pattern was revealed, with central necrosis confirmed by pathological examinations. Central necrosis was also observed in other graft models (7, 10-12). The global tumoral volume increased 6.5-fold between days 14 and 30, this was essentially bound to the appearance of central necrosis. In fact, there was no necrosis on day 14 and 6.305 cm³ of necrosis on day 30. The metabolic tumoral volume increased slowly, 1.265 to 1.9625 cm³ between days 14 and 30. In our experimentation, we did not observe any visceral FDG uptake, confirmed by pathological examination. FDG-PET/CT is a valuable tool for staging the tumor and detecting metastasis (7, 13). This imaging was demonstrated to be feasible, of excellent quality and quite quantitatively accurate for research in rats with tumors of appropriate size superior to 1 cm³. This imaging is also interesting for following tumor progression and for evaluating therapeutics efficacy (14, 15). By using 18F-FDG to monitor treatment, accurate intratumoral distribution information would permit a better understanding of therapy efficacy which may prove helpful for a better evaluation of therapy.

We have established by allograft the first HNSCC in immunocompetent rats. It is a realistic and reproducible model that could be used for various studies, even therapeutic tests.

References


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