The Auricular VX2 Carcinoma: Feasibility of Complete Tumor Resection

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Abstract. Background: The aim of the study was to test the feasibility of resection of VX2 auricular squamous cell carcinoma (SCC) in New Zealand White rabbits (NZW), its influence on the regional and distant metastases and the general prognosis. Materials and Methods: In 71 NZW rabbits, successful in sano wide margin resection of auricular VX2 SCC was performed 7 days after tumor induction. The animals were sacrificed and examined for metastases after 6 weeks. Results: Local recurrences occurred in 7% of the animals. In 28.2% lymph node (LN) and in 15.5% distant metastases developed. Regional metastases presented in 100% of the animals with recurrence and in 22.7% of the R0 animals (p<0.001). Distant metastases presented in 80% of the animals with local recurrence and in 10.6% of the R0 animals (p<0.001). Of the animals with local control, those with N+ had a significantly higher incidence of distant metastases (46.7%) compared to those with N0 (0%, p<0.001). Local control and LN involvement were found to be important prognostic factors for early tumor-related death. Conclusion: The VX2 auricular model resembles head and neck (HN) SCC in humans morphologically, pathophysiologically and as a complex neoplastic disease. In the experimental setting it behaved similarly to the clinical situation of wait-and-see in N0 HN SCC patients. Tumoral microemboli present a possible mechanism for the development of recurrences.

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Key Words: VX2, squamous cell carcinoma, metastases model, tumor resection.
Materials and Methods

The animal use protocol was approved by the Institutional Animal Care Use Committee of the government of Giessen, Germany. The experiments were performed in accordance with the guidelines of the Declaration of Helsinki and in accordance with the Public Health Service Policy of Humane Care and Use of Laboratory Animals.

The VX2 cell line was propagated in vivo by intramuscular passage in the gluteal musculature of NZW rabbits. From these tumors, standard cell suspensions with mean densities of $8 \times 10^6$ vital cells/ml, tested by Trypan blue exclusion, were prepared. In 73 adult male specific pathogen-free Ifa Credo NZW (ICO:NZW) rabbits weighing $3.0 \pm 0.6$ kg on day 0, auricular tumors were induced by injecting 0.3 ml of the suspension between the central auricular neuro-vascular bundle and the caudal margin on the dorsal side of the auricle. The animals were followed daily over the next 7 days, and body weight, rectal temperature and tumor size in 3 planes were measured. The macroscopic aspect of the tumor was estimated with semi-quantitative scales for tumoral bleeding, necrosis and crust. The neck status was examined by palpation. The general condition of each animal, the quantities of water and food consumed and feces and urine discharged were monitored daily. The resection was carried out on the 8th day after the tumor inoculation. The animals were anesthetized with 5 mg/kg xylazinhydrochloride (Rompun® 2%, Bayer Vital GmbH, Leverkusen, Germany) and 100 mg/kg ketaminhydrochloride (Ketavet®, Pharmacia GmbH, Erlangen, Germany). The medial auricle artery was ligated. The resection line was placed well proximal to the inferior tumor margin (1.5 cm). The wound was covered with skin flaps and the sutures were removed on the 7th postoperative day. The animals were sacrificed on the 42nd postoperative day using an intravenous application of 1 ml T-61 (Intervet®, German GmbH, Unterschleissheim, Germany), after being anesthetized.

For an evaluation of the extent of metastatic spread to the regional lymph nodes (LNs), the ipsilateral neck side was dissected, and the organs of the thoracic and abdominal cavities were examined for distant metastases. The number and size of metastases were recorded. The specimens were further processed for histological preparation (hemalaun and eosin stain).

For the statistical analysis, SPSS for Windows v. 11.5 (SPSS Inc., Chicago, IL, USA) was used. Categorical data were analyzed using cross-tabulation and the Chi-squared test for independence. In cases of small samples, the Fisher’s exact test was used. For comparison of nominal-level variables between groups, the unpaired \( t \)-test was used. For statistical significance, two-tailed test was used. Probability levels less than 0.05 were considered statistically significant. Relationships were analyzed with the Pearson product moment correlation coefficient.

Results

Of the 73 animals, 2 were lost to anesthesia problems and were thus excluded from further analysis. The tumor inoculation was successful in all animals. The absolute dimensions of the tumoral masses increased clearly over the 7 days before resection. The tumor growth, which is a better measure of the tumoral viability and aggressiveness than the absolute dimensions, was estimated by the percentage increase of the tumoral dimensions (Table I).

Of the 71 animals which survived, intervention in 7% local recurrences occurred, consisting, in all cases, of a single solid tumoral mass situated on the tumoral side of the auricle in the area of ligation of the central auricular vessel bundle. The recurrences became clinically evident during the 2nd postoperative week in 2/5 and during the 5th week in 3/5 animals. At the point of sacrifice, recurrences had mean longitudinal diameters of $23.2 \pm 18.5$ mm and transversal diameters of $18.7 \pm 14.7$ mm.

Twenty animals (28.2%) had LN metastases. In 20/20 there was metastatic involvement of the first echelon parotid LN - level I (Figure 1). One animal had 2 clearly delimited tumoral masses in the parotid region, each with thick capsule. In 4/71 animals (5.6%) further LNs were also involved. In 3 animals the metastases were found in the LNs of the caudal mandibular group (level II), and in 1 animal metastases were found in the LNs of the rostral mandibular group (level III). All animals with metastases in levels II and III also had metastases in level I. All animals with recurrences at the primary site had LN metastases; all of them presented with metastatic involvement of the first echelon parotid LN (level I) and 2/5 (40%) of a second LN from the caudal mandibular group (level II). Of the 66 animals, proven to be tumor-free at the primary site after resection (complete local control), LN metastases were present in 15 (22.7%). In 15/15 there was metastatic involvement of the first echelon parotid LN (level I). In 2/66 (3.0%) more distant LNs were also involved; in 1 case a LN from the caudal mandibular group (level II) and the other a LN from the rostral mandibular group (level III). Compared to the incidence of LN metastases by the Fisher’s exact test, the group with successful R0 resection had significantly fewer metastases in the level I LNs (\( p=0.001 \)) and to other consecutive levels (\( p=0.02 \)) than the group with local recurrences (Figure 2).

At dissection the abdominal and thoracic organs were examined for macroscopic metastases. In no cases were distant metastases observed at locations other than the lungs, and the incidence of distant metastases was 11/71 (15.5%). The mean number of unique seedings per animal varied from 1 to 69 (in both lungs), with a mean value of

### Table I. Dimensions and growth of the tumor.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Day 1 (mm)</th>
<th>Day 8 (mm)</th>
<th>Growth (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor length</td>
<td>9.6±1.9</td>
<td>18.1±4.3</td>
<td>192.3±53.8</td>
</tr>
<tr>
<td>Tumor width</td>
<td>7.4±1.8</td>
<td>13.2±3.1</td>
<td>184.7±56.4</td>
</tr>
<tr>
<td>Tumor thickness</td>
<td>3.3±0.6</td>
<td>7.0±2.5</td>
<td>217.8±80.7</td>
</tr>
</tbody>
</table>
15.5±19.1. Animals with tumoral recurrence had significantly higher incidence of lung metastases (4/5, 80%) than those with local control (7/66, 10.6%), p=0.02. Animals that had no local tumor recurrence and only regional LN metastases had significantly higher incidences of lung metastases (7/15, 46.7%) than animals without regional tumor disease (0/51, 0%), p<0.001. All animals with lung metastases also had neck LN metastases. No cases with skip hematogenous metastases from the primary tumor to distant organs were observed. The total number of lung metastases did not differ significantly between the group with tumor recurrence (8.8±6.9) and the group with local control (19.3±23.2).

The overall prognosis was estimated based on two clinical parameters: early tumor-related death or tumor-related worsening of the general condition. The mean follow-up was 5.9±0.3 weeks (range 4-6). Four animals died before the planned time-point of tumor progression 45±2.7 (38-50) days after tumor induction. Two of them had local recurrence, and the other 2 had local control, but all 4 had LN involvement and 3/4 had distant metastases. An important parameter of the animals’ general condition, apart from the signs of local or regional tumoral involvement, was the body mass. The animals were fed *ad libitum*, and the general trend was towards increase. The percentage weight gain at the end-point compared to the start was 9.1%±10.9 (range –23.8 to 45%). The weight gain in the tumor-free animals was significantly higher than in those with tumors (11.3%±9.4 versus 3.4%±12.7, p=0.005).

**Discussion**

The VX2 auricular carcinoma model has similarities with human SCC with regard to morphological and pathophysiological aspects, as well as the nature and progression of the disease. Histologically, the model presents a typical SCC with a very low differentiation and anaplastic appearance. The cells are highly pleomorphic, and are polygonal, contain large cytoplasm and enlarged nuclei. The cell borders are usually well delineated and sharp and the nucleus is centrally located. Mitotic figures are commonly observed. The prominent nucleoli and the mitotic figures prove the low differentiation of the tumor.

The rabbit’s ear has several advantages as a primary tumor site. It is easily accessible for observation, measurements and manipulation and, in our experimental setting, this allowed the resection to be performed in a...
uniform way without being limited by the endoscopic approach, as with intraoral implantation. Since the ear is a periphery structure, far from important organs, vessels and nerves, tumor growth and manipulations on it cause less discomfort to the animals.

The metastatic tumor spread of the VX2 auricle SCC initially occurs along the lymphatic vessels, affecting a well-studied cascade of LNs. At later stages the metastatic process involves distant sites – namely the lungs. This chronologically and topographically reproducible biological process is analogous to the process of regional and distant metastatic spread of human HNSCC (2, 16). Here, every tumor location in the upper aerodigestive tract corresponds to certain regional lymph nodes that are usual sites of metastases from these primary locations (20-22). Apart from this lymphatic spread, tumoral cells may further propagate from the primary site and the involved LN to distant locations (usually the lungs, liver or bones) via the blood circulation (2, 23). The VX2 auricle SCC in NZW appears a successful model for the pathophysiological mechanisms of metastatic spread.

In addition to the morphological and pathophysiological aspects discussed, the VX2 SCC in NZW behaved, in general, as a typical neoplastic disease in its entire complexity. Our experimental setting is analogous to the clinical situation with resected primary tumors and the "wait and see" approach to the neck. The neck metastases in the R0 group showed background dissemination after tumor induction. The results showed that, on day 8, metastatic spread has occurred in about 23% of the animals, though still not clinically evident. The incidence of LN metastases in the VX2 auricle SCC model has been shown to be independent from the primary tumor inoculation technique (2). In this way, the VX2 auricle model corresponds to the natural history of HNSCC in humans, where the important factors related to metastatic spread are tumor size and depth of infiltration (23-25). Newer analyses point out that tumor thickness rather than tumor size is related to the incidence of regional metastases (26, 27). In our VX2 model, a slightly significant correlation was found only between the maximal width of the tumor and the presence of LN metastases ($r=0.18, p=0.038$). The other tumor dimensions (maximum length, thickness, base surface area), as well as the increase of all these dimensions from day 1 to day 9, did not seem to correlate with the presence of regional metastases.

The observed local recurrences, quite unexpected at the initial planning of the experiment, are of special interest. They could hardly be attributed to the surgical technique since the resection was performed in sano with a large margin. Field cancerization should be excluded as a possible cause for the recurrences, as this is a xenotransplant cancer model, where the tumor is implanted in previously normal tissue. Microscopic progression per continuitatem at 1.5 cm from the visible tumor border is also unlikely. The histological slides showed a clear border between the tumor and the healthy tissue. The tumor did not show a tendency to infiltrate or give projections far away in the periphery (per continuitatem). The most probable mechanism appears to be short distance metastasis along the lymphatic/blood vessels. The VX2 SCC grows as a solid tumor with an active periphery, where the tumor gives small emboli of multiple, well-adhered cells. These emboli could be found in the lymphatic as well as in the blood vessels (Figure 3). Stasis in the area, caused by tumor manipulation or vessel ligation, could lead to an end of these emboli and help their local adhesion. In this way, local metastatic colonization develops. In our experimental setting, the time interval between the resection and the clinical evidence of the recurrent tumoral mass was quite variable. The most early recurrences were detected 14 days after the resection, the latest one 28 days after the resection. Such a late appearance of recurrences shows that the tumoral cells could survive for a significant time in the host before they start to proliferate.

The relationships between the incidence and characteristics of the lung metastases and some clinical and pathological factors were analyzed with the Pearson product moment correlation coefficient. The presence of distant metastases correlated significantly with the local recurrences ($r=0.49, p<0.001$), with the involvement of the first echelon LN ($r=0.68, p<0.001$) and further nodal involvement ($r=0.4, p=0.001$). In order to check which one of these factors could be the main indicator for distant metastases, partial correlation and linear regression analysis were performed. After controlling for any one or a combination of these variables again, all were found to correlate with pulmonary involvement. This means that, in our experimental setting, both the local control (R0 resection) and the regional involvement were equally important factors for distant tumoral spread. On the other hand, distant metastases seemed not to be related to the tumor growth speed and maximal size. These finding correspond to the results of other investigators (2).

The correlation between the presence of lung metastases and poor prognosis (early death) was moderate ($r=0.55, p<0.001$). There was a very high negative relationship between the number of pulmonary metastases and survival ($r=-0.92, p<0.001$). Weight loss appeared to be strongly associated with the presence of distant metastases, a phenomenon also observed in humans with end-stage disease. For the VX2 model, van Es et al. reported a significant correlation between weight loss and primary tumor size and between weight loss and the size of lung metastases (2). Weight loss after the 3rd postoperative week was a significant predictor for the presence of lung metastases ($p<0.01$).
Conclusion

The aim of the present study was to evaluate a standard therapeutic approach (resection) with curative intent in the VX2 auricle squamous cell carcinoma model in NZW rabbits. Quantitative estimation of local and regional control and systemic neoplastic disease was carried out. Apart from the morphological similarity with human HNSCC and the concordance with the mechanism of metastatic spread to predefined lymphatic regions, the model showed natural disease progression and response to therapy, which again confirmed those observed in human HNSCC. For the first time, local recurrences of VX2 carcinoma were observed, most probably appearing as a result of microembolization. The recurrences proved to be a decisive factor for both lymphatic and hematogenous metastatic spread and the overall prognosis. The achievement of complete local control over the primary site appeared to be most important therapeutic goal. The estimated incidence of local recurrences, regional lymphatic and distant hematogenous spread could serve as reference values for evaluating the feasibility of other therapeutic approaches. The possibility to simulate neoplastic disease under uniform conditions, with all its stages from tumor induction to end-stage disease over 7 weeks, could allow for the rapid evaluation of new experimental treatment modalities.

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References


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