Brachytherapy for Oral Tongue Cancer: An Analysis of Treatment Results with Various Biological Markers

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Objective: Low-dose-rate (LDR) brachytherapy is an effective treatment for tongue cancer. However, little is known about the biological mechanism underlying this therapy, characterized by delivery of continuous exposures of LDR irradiation. It is reported that lower microvessel density (MVD), lower Ki-67 index or higher expression of endogenous hypoxic markers such as carbonic CA IX and Glut-1 are related to the poor control of tumors treated with external irradiation. To elucidate the biological characteristics of LDR brachytherapy, we analyzed our results in cases of tongue cancer treated with LDR brachytherapy by using immunohistochemical stainings with antibodies against Ki-67 and MVD, Glut-1 and CA IX.

Methods: The prognostic value of Ki-67 index, MVD and the expression of CA IX and Glut-1 was assessed in 68 tongue cancers treated with LDR brachytherapy. The specimens were taken from tongue cancers before radiation therapy and immunohistochemical staining was performed.

Results: The local recurrence-free survival rates were significantly different between T1 + T2 and T3 (P = 0.00067), but not between low and high Ki-67 indexes (P = 0.54), between low and high MVD (P = 0.071), low and high CA IX indexes (P = 0.062) or low and high Glut-1 indexes (P = 0.107). T stage, the size of the tumor was the only significant factor for local control in multivariate analyses (P = 0.0377).

Conclusion: LDR could overcome the radioresistance of non-cycling and hypoxic cells; however, we cannot draw firm conclusions due to the limited number of patients.

Key words: tongue cancer – brachytherapy – Ki-67 – microvessel density – CA IX

INTRODUCTION

Low-dose-rate (LDR) brachytherapy with or without external irradiation provides a high local control rate in the treatment of early tongue cancer, comparable to that obtained by surgery (1–3). Recently, LDR brachytherapy has been performed in prostate cancer with excellent results, and the number of patients treated with LDR brachytherapy has been increasing (4).

The good dose distributions delivered by radioactive sources in or near the tumor are a reason for the high local control rate obtained by brachytherapy. In addition, however, LDR brachytherapy is characterized by delivery of continuous exposures of LDR irradiation, which might produce biological advantages that external irradiation does not possess.

Although many in vitro and in vivo researches about the biological effects of continuous LDR have been performed (5), there are no reports on LDR research in which clinical specimens were used for immunohistochemical analysis as far as we are aware.

In this report, in order to elucidate the biological characteristics of LDR brachytherapy, we analyzed our results in cases of tongue cancer treated with LDR brachytherapy, by using immunohistochemical stainings with antibodies against Ki-67 and microvessel density (MVD), Glut-1 and CA IX.

MATERIALS AND METHODS

POPULATION

Between 1987 and 2004, 78 patients with Stage I–IV squamous cell carcinoma of the oral tongue, according to the 1997 International Union Against Cancer TNM...
Classification, were treated radically with radiotherapy alone. Biopsy samples of four patients were lost or scanty, and six patients who received both external beam irradiation and LDR brachytherapy were excluded from this analysis; 68 patients were, therefore, retrospectively selected.

Patient characteristics are shown in Table 1. The follow-up period ranged from 11 to 146 months (mean 56 months, median 54 months). The minimum follow-up period of the other living patients was 28 months.

**TREATMENT**

All 67 patients were treated by LDR brachytherapy alone. The technique of implantation for brachytherapy is same as that in the Manchester system (6,7). Cesium 137 needles were used as radioactive sources. The prescribed dose was 65–70 Gy over a period of 6–7 days when LDR brachytherapy alone was used. No patient had received chemotherapy before or during radiotherapy.

Nine patients had positive neck lymph nodes (N1 or N2). These patients did not hope to be treated with glossectomy. The radical or modified neck lymph node resection was performed after LDR brachytherapy to the primary tumor.

**IMMUNOHISTOCHEMICAL EXAMINATION**

One to three biopsies, 1–5 mm in diameter, were taken from each tumor. All of the biopsies were taken at the initial time of the diagnosis. Immunohistochemical staining was carried out with the methods previously described (8).

Immunohistochemical detection for growth fraction was performed with Ki-67 staining using MIB-1 monoclonal antibody (DAKO, Copenhagen, Denmark) and that for MVD with CD34 monoclonal antibody (Nichirei, Japan).

Rabbit polyclonal antibody to Glut-1 (Chemicon International, USA) and that to CA IX (Novus Biologicals, CO, USA) were also used.

**EVALUATION OF IMMUNOSTAINING**

The percentages of Ki-67-positive tumor cells were calculated by counting the number of brown-stained tumor nuclei/totai number of cancer cells in the most highly stained area, with a highly magnified view (×400; 0.196 mm²/field). More than 400 cells were counted in each specimen. The Ki-67 labeling index (Ki-67 index) was estimated by the percentage of Ki-67-positive cancer cells among all the tumor cells counted.

The microvessel count was assessed by light microscopy in three of the most extensive areas of neovascularization (termed 'hot spots') with a highly magnified view (×400; ×40 objective and ×10 ocular; 0.196 mm²/field), and the average number of vessels was calculated. We counted intratumoral and stromal vessels with actual lumens around the tumor nests, but did not count a single endothelial cell (or cluster) or vessels that existed far from the tumor nests (9,10).

In evaluation of CA IX and Glut-1 positivity, the specimens were scanned at low optical power (×40 and 100), and the percentage of cells with positive Glut-1 or CA IX reactivity was assessed (11).

Measurements of immunostaining of these proteins were performed independently in all cases by two investigators who had no previous knowledge of the clinical outcome. When the evaluation for each antibody differed between investigators, the investigators discussed it, with or without re-evaluation, until an agreement was reached.

**STATISTICAL ANALYSIS**

Local recurrence-free rates in patients were measured using the Kaplan–Meier method. Differences were analyzed by the log-rank test with significance taken at $P < 0.05$.

Differences in various markers between local control and local failure were analyzed by $t$-test (two-sided) with significance taken at $P < 0.05$. Patients who died < 24 months after LDR brachytherapy were not included in local control, even if they had local control until their death. Multivariate analysis was also performed using Cox’s proportional hazard regression model (12).

The date of diagnosis was defined as the date of biopsy confirmation of disease, and survival was calculated from this date to the time of death or last follow-up.
RESULTS

KI-67 INDEX AND LOCAL CONTROL

Figure 1a shows Ki-67 positive cells immunohistochemically detected using anti-Ki-67 antibody. Table 2 shows the relationship between the Ki-67 index and local control for tongue cancer treated with LDR brachytherapy.

The Ki-67 index ranged from 13 to 75%. The mean Ki-67 index for the local control group was 40% and that for the local failure group was 37%. There was no correlation between Ki-67 index and local control ($P = 0.59$).

In order to clarify whether the Ki-67 index is related with local control rate, tumors were divided into two groups (high Ki-67 index and low Ki-67 index) and the local recurrence survival rates were compared (Fig. 2a). There was no correlation between the Ki-67 index and local control ($P = 0.54$).

MVD AND LOCAL CONTROL

Figure 1b shows the microvessels immunohistochemically detected using anti-CD34 antibody. Table 2 shows the relationship between MVD and local control. MVD ranged from 5 to 45 microvessels per field (50.391 mm$^2$). The mean MVD for the local control group was 21 vessels/field and that for the local failure group was 20 vessels/field. There was no correlation between MVD and local control ($P = 0.52$).

In order to clarify whether MVD is related with local control rate, tumors were divided into two groups (high MVD and low MVD) and the local recurrence survival rates were compared (Fig. 2b). There were no significant

Table 2. Relationship between the expression of various markers and the local control for tongue cancer

<table>
<thead>
<tr>
<th>Markers</th>
<th>Mean (%)</th>
<th>Range (%)</th>
<th>$P$ value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki-67 index</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Local control</td>
<td>40</td>
<td>13–75</td>
<td>0.59</td>
</tr>
<tr>
<td>Local failure</td>
<td>37</td>
<td>17–58</td>
<td></td>
</tr>
<tr>
<td>Microvessel density</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Local control</td>
<td>21*</td>
<td>5–45*</td>
<td>0.52</td>
</tr>
<tr>
<td>Local failure</td>
<td>20*</td>
<td>5–27*</td>
<td></td>
</tr>
<tr>
<td>CA IX expression</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Local control</td>
<td>10</td>
<td>0–60</td>
<td>0.56</td>
</tr>
<tr>
<td>Local failure</td>
<td>12</td>
<td>0–50</td>
<td></td>
</tr>
<tr>
<td>Glut-1 expression</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Local control</td>
<td>30</td>
<td>0–80</td>
<td>0.62</td>
</tr>
<tr>
<td>Local failure</td>
<td>18</td>
<td>0–80</td>
<td></td>
</tr>
</tbody>
</table>

*The $P$ value of the statistical difference between local control and local failure.

# The number of microvessels/field.

CA IX, carbonic anhydrase 1%.

Figure 1. Representative immunohistochemical stainings for Ki-67, CD34, CA IX and Glut-1 in biopsy specimens of patients with tongue cancer. (a) Ki-67, in this case, nuclear staining is scattered in the specimen, ×400. (b) CD34 for microvessels. The microvessels are most numerous at the periphery of the tumor, ×200. (c) CA IX, diffuse membrane expression was noted, ×200. (d) Glut-1, diffuse membrane expression was noted, ×200.
differences in local control between tumors with high MVD and those with low MVD ($P = 0.071$).

**CA IX EXPRESSION AND LOCAL CONTROL**

Figure 1c shows the immunostaining of CA IX. Table 2 shows the relationship between CA IX expression and local control. The percentage of cells with positive CA IX reactivity ranged from 0 to 60%. The mean CA IX positivity for the local control group was 10% and that for the local failure group was 12%. There was no correlation between CA IX expression and local control ($P = 0.555$).

In order to clarify whether CA IX positivity is related with local control rate, tumors were divided into two groups (low/negative CA IX and high CA IX expression) and the local recurrence survival rates were compared (Fig. 2c). We used the same cutoff point (<10 versus >10%) as Koukourakis et al. (11) to define two groups of tumors with low/negative and high CA IX reactivity. There were no significant differences in local control between patients with negative/low CA IX expression and those with high CA IX expression ($P = 0.062$).

**GLUT-1 EXPRESSION AND LOCAL CONTROL**

Figure 1d shows the immunostaining of Glut-1. Table 2 shows the relationship between Glut-1 expression and local control. The percentage of cells with positive Glut-1 reactivity ranged from 0 to 80%. The mean Glut-1 positivity for the local control group was 30% and that for the local failure group was 18% (Table 2). There was no correlation between Glut-1 expression and local control ($P = 0.62$).

In order to clarify whether Glut-1 expression is related with local control rate, tumors were divided into two groups (high Glut-1 expression and Glut-1 expression) and the local recurrence survival rates were compared (Fig. 2d). There was no significant correlation between Glut-1 expression and local control ($P = 0.11$).

**T STAGE AND LOCAL CONTROL**

The actuarial local recurrence-free rates at 5 years were 100, 85.5 and 22.2% in the patients with T1, T2 and T3 tumors, respectively (Fig. 2e). A significant difference was found in the 5-year local recurrence-free rate between T1 + T2 and T3 tumor groups ($P = 0.00067$).

T stage had no significant correlation with Ki-67 ($P = 0.55$), CA IX ($P = 0.63$), Glut-1 ($P = 0.12$) or MVD ($P = 0.051$).

**MULTIVARIATE ANALYSES FOR LOCAL CONTROL**

Ki-67 index, MVD, expression of CA IX, expression of Glut-1 and T stage were analyzed for prognostic significance in local control by multivariate analysis. T stage
Table 3. Multivariate analysis of factors prognostic for local recurrence-free survival

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard ratio</th>
<th>95% CI</th>
<th>P value</th>
<th>Better prognosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>T stage (T1, T2 versus T3)</td>
<td>0.00133</td>
<td>0.0000026–0.685</td>
<td>0.0377</td>
<td>T1, T2</td>
</tr>
<tr>
<td>Ki-67 index (38≥ versus &lt;38)</td>
<td>0.588</td>
<td>0.0514–6.74</td>
<td>0.7</td>
<td>38≥</td>
</tr>
<tr>
<td>CA IX expression (10≥ versus &lt;10)</td>
<td>1.92</td>
<td>0.222–16.6</td>
<td>0.554</td>
<td>10≥</td>
</tr>
<tr>
<td>Glut-1 expression (27≥ versus &lt;27)</td>
<td>1.11</td>
<td>0.153–8.12</td>
<td>0.914</td>
<td>&lt;27</td>
</tr>
<tr>
<td>MVD (21≥ versus &lt;21)</td>
<td>0.798</td>
<td>0.147–0.685</td>
<td>0.794</td>
<td>21≥</td>
</tr>
</tbody>
</table>

CI, confidence interval.

*aThis index was divided above or below the average value.

(P = 0.0377) was the only significant prognostic factor in local control. Ki-67 index, MVD, expression of CA IX or expression of Glut-1 had no significance in local control of tongue cancer (Table 3).

DISCUSSION

In general, radiation kills proliferating tumor cells more efficiently than quiescent tumor cells, resulting in many clonogenic tumor quiescent cells remaining following radiotherapy (13–15). Therefore, it is thought to be harder to control quiescent tumor cells than to control proliferating tumor cells, and many post-radiotherapy recurrent tumors are thought to result partly from the re-growth of quiescent tumor cell populations that were not killed by radiotherapy (16).

Ki-67 is a nuclear protein which is expressed in cycling cells. For patients treated by radiotherapy, the tumors with high Ki-67 labeling index showed good local control in squamous cell carcinomas of the head and neck (17) and esophageal cancer (18), uterine cervical cancer (19) and bladder cancer (20). However, there was no such relationship between the Ki-67 labeling index and local control of tongue cancer treated with LDR brachytherapy in our results. This indicated that LDR brachytherapy may be as effective for tumors that included more non-cycling cells as it was for tumors that included more cycling cells. The accumulated dose over the cell cycle is an appropriate indicator of cell lethality with continuous irradiation (5). A given dose rate of continuous irradiation is more damaging to cells with long cell cycles, because a larger dose is absorbed in each cell cycle. Therefore, non-cycling cells that have much longer cell cycles receive a larger dose over the cell cycle and may be killed more effectively with LDR brachytherapy.

Based on the in vivo observations of vascular geometry and blood flow in the tumor microcirculation, oxygen delivery to tumor tissues appears to rely on a network of microvessels indicating that tumor angiogenesis correlates with the oxygenation of tumor tissue. Hulka et al. (21) and Secomb et al. (22) reported that MVD correlates well with blood flow in breast tumors. Tumor MVD was introduced as a representative of O2 status in laryngeal carcinoma (10) and esophageal cancer (9). In T1- and T2-stage laryngeal carcinoma treated with radiotherapy, multivariate analysis and Kaplan–Meier analysis showed that MVD alone had significant predictive power for radiosensitivity. They concluded that MVD was a useful predictive marker for evaluating radiosensitivity in laryngeal carcinoma (10).

The facilitative glucose transporter 1, Glut-1 is up-regulated via an oxygen-sensing pathway involving the hypoxia-inducible factor-1α (HIF-1α), a transcription factor that is expressed in most cells in response to hypoxia (23,24). CA IX is a novel member of the carbonic anhydrase (CA) family that codes for a transmembrane glycoprotein that is expressed in most cells in response to hypoxia (23,24). CA IX is a novel member of the carbonic anhydrase (CA) family that codes for a transmembrane glycoprotein and is also an HIF-1α-dependent gene (25,26). In head and neck cancer treatment with external irradiation, patients with high expression of Glut-1 or CA IX had significantly poorer results in local control than those with the low/negative expression (11,27,28).

In order to analyze the influence of hypoxia on local control of tongue cancer treated with LDR brachytherapy, we investigated MVD and the expression of endogenous hypoxic markers such as Glut-1 and CA IX in tongue cancer tissues. In contrast with the results of external irradiation, MVD and the expression of hypoxic markers had no relationship with local control. These results indicate that LDR brachytherapy might achieve an equally successful cure of hypoxic tumors as obtained with oxyic tumors. In LDR brachytherapy, the repair of sublethal damage occurs during a long period of radiation exposure. In vivo experiments demonstrated that sublethal damage repair is an active process requiring oxygen and nutrients (5). Therefore, hypoxic tumor cells could be killed more effectively than oxyic tumor cells with LDR brachytherapy due to lower sublethal damage repair.

Inoue et al. (29) reported that hyperfractionated high-dose-rate (HDR) brachytherapy for early mobile tongue cancer has the same local control compared with LDR brachytherapy. The good results of HDR brachytherapy in our study may be due to the good dose distributions delivered by radioactive sources in or near the tumor because HDR does not have the biological benefits that LDR has. However, HDR caused bone exposure as an adverse effect. Since the number of patients in the study was small, further study of HDR may be required.

In conclusion, T stage, the size of the tumor was the only significant factor for local control of tongue cancers treated with LDR brachytherapy. The Ki-67 index, MVD and expression of endogenous hypoxic markers such as CA IX and Glut-1 had no correlation with local control. These results indicate that LDR may overcome the radioresistance of hypoxic cells and non-cycling cells although we cannot draw conclusions due to the limited number of patients. Such advantages of LDR brachytherapy over external
irradiation might be responsible for the better results of LDR brachytherapy.

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Conflict of interest statement

None declared.

References


