Image Analysis of Microvessel Surface Area Predicts Radiosensitivity in Early-Stage Laryngeal Carcinoma Treated with Radiotherapy

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ABSTRACT

Purpose: The tissue oxygenation level, which is theoretically governed by distance from blood vessels, is one of the most important modulators of the radiosensitivity of carcinoma. A computed image analysis system for the detection of tissue oxygenation was developed to establish a method of predicting radiosensitivity in early-stage laryngeal carcinoma treated by curative radiotherapy.

Experimental Design: Microvessel structures labeled with CD31 antigen were investigated in 55 patients undergoing curative radiotherapy for T1 and T2 laryngeal carcinoma. We calculated (a) microvessel density [(MVD) vessels/field] under a microscope; (b) the ratio of the total microvessel number (TN):tumor area (TA) [TN:TA; vessels/mm²]; (c) the ratio of the total microvessel perimeter (TP):TA (TP:TA; mm/mm²); and (d) the ratio of tumor tissue area >150 μm from microvessels (hypoxic ratio; %) as parameters of tissue oxygenation in each whole biopsy specimen by using an image analyzer. We compared each of these factors with radiosensitivity.

Results: Mann-Whitney’s U test revealed that tumors with a high MVD (median, 42 vessels/field), high TN:TA ratio (median=40.9 vessels/mm²), high TP:TA ratio (median, 2.92 mm/mm²), and low hypoxic ratio (median, 30.3%) had significantly greater radiosensitivity than tumors with a low MVD, low TN:TA ratio, low TP:TA ratio or high hypoxic ratio (P = 0.002, P = 0.0004, P < 0.0001, and P = 0.004, respectively).

Conclusions: Prediction of radiosensitivity on the basis of the TP:TA ratio can be used as an efficient means of avoiding ineffective radiation, complications after salvage surgery, and prolonged hospital stays.

INTRODUCTION

Radiotherapy is generally considered to provide a fair clinical outcome with organ preservation in laryngeal carcinoma patients. Total or partial laryngectomy is performed as salvage therapy in most patients with residual or recurrent disease. Such patients require more aggressive treatment and longer hospital stays, and have poorer quality of life, because radiotherapy causes delayed operative wound healing, and, thus, there is a desire for new biological markers that predict tumor radiosensitivity. Several parameters, including proliferative activity and detection of products of apoptosis-associated proteins, have been suggested as prognostic indicators. However, controversial results regarding the significance of the expression of these factors have been reported to date (1–3).

The basic radiation biology of tumor tissues, both in vivo and in vitro, indicates that well-oxygenated cells are fully radiosensitive (4–6). If tumors that contain viable hypoxic tumor cells could be identified, the efficiency of radiotherapy might be easily evaluated. We recently assessed predictive factors for the radiosensitivity of laryngeal carcinomas and found that MVD3 at the hot spot in the tumor is a potentially useful factor for predicting radiosensitivity (7). Hulka et al. (8) and Secomb et al. (9) reported that the MVD of tumors is correlated with extraction-flow products measured by magnetic resonance imaging (MRI). This finding combined with our previous data indicates that tissue oxygenation represented by MVD is a good predictor for the radiosensitivity of laryngeal carcinomas.

Our previous study, however, contained two methodological limitations. First, the selection of areas of maximum vascularization in the so-called “hot spot approach” to determining MVD is prone to error because of inter- or intraobserver bias. Second, the assessment of vessel counts on virtually two-dimensional histological sections is influenced by the coiling, tortuosity, and compression of vessels. Thus, vessel counts in hot spots for MVD have been performed without taking vessel shape and size into consideration, although the surface of larger vessels may have better ability to diffuse oxygen than that of smaller vessels. To evaluate tissue oxygenation in tumors more accurately and to overcome the limitations of using biopsy...
specimens, we tried assessing all of the microvessels in entire biopsy specimens by using computed image analysis. The development of this new method of image analyzing using conventional microscopes minimizes intra- and interobserver bias and heterogeneity of biopsy specimens.

Oxygen delivery to tumor tissue appears to depend on diffusion from microvessels. The perimeter of the microvessel on sections theoretically represents the surface area of microvessels that can provide for tissue oxygenation. Eric (10) calculated that the distance of oxygen diffusion into tumor tissue around vessels is \( \sim 150 \mu m \). In addition to \((a)\) MVD, we developed new methods of analysis that consist of calculating \((b)\) the ratio of total microvessel number (TN):total tumor area (TA; TN:TA ratio; vessels/mm\(^2\)); \((c)\) the ratio of total microvessel perimeter (TP) to total tumor area (TA; TP:TA ratio, mm/mm\(^2\)); and \((d)\) the ratio of tumor tissue area (hypoxic ratio, \%) by image analysis. We compared the factors to determine which, in addition to clinicopathological factors, is the most sensitive predictor of radiosensitivity in T\(_1\) and T\(_2\) laryngeal carcinoma.

### MATERIALS AND METHODS

**Patients.** We reviewed the medical records of 85 patients who had undergone radical radiotherapy for T\(_1\) and T\(_2\) stage laryngeal carcinoma at the National Cancer Center Hospital East (NCCH) between September 1992 and November 1997. The study was limited to patients whose lesion had been biopsied and whose outpatient follow-up period at NCCH had been \( \geq 3 \) years. Follow-up examination were performed every 1–3 months during the 1st year after radiotherapy, every 3–6 months during the next 3 years, and annually thereafter. Fifty-five patients were ultimately selected for the study. They had received radiotherapy alone at a total curative dose of 52–70 Gy (52 Gy: one case; 60 Gy: 15 cases; 66 Gy: 11 cases; 70 Gy: 28 cases). The clinicopathological characteristics of all of the cases are listed in Table 1. All of the patients were irradiated with one daily fraction Monday through Friday at a dose rate of 1 Gy.

Tumor size evaluated on the basis of the laryngoscopic images ranged from \( \sim 4 \) to 15 mm in diameter. All of the 55 biopsy specimens used in this study were collected at the time of initial diagnosis. One to three specimens were taken from each tumor, and the size of the specimens ranged from 2 to 5 mm in diameter. All of the patients except one were habitual smokers.

**Histological differentiation** were performed according to the classification of the Union International Contre le Cancer (UICC; Ref. 11). Nineteen (34.5%) of the 55 patients developed recurrences at the primary site, and all of the recurrent tumors were histologically confirmed. Eight patients with recurrent tumors underwent total laryngectomy, nine underwent partial laryngectomy, one underwent laser surgery, and one patient refused salvage surgery. The mean interval until the start of oral feeding in the eight patients who underwent total laryngectomy was 103.1 days (range, 13–175 days). We defined a tumor as “radiosensitive” if it completely disappeared from the primary region for at least 3 years, and as “radioresistant” if there was a residual tumor or a recurrent tumor within 3 years after irradiation.

**Immunohistochemical Staining.** Microvessels were detected by using a monoclonal antibody against the CD31 antigen (clone JC70; Dako, Glostrup, Denmark). The primary antibody was diluted to 1:200 in 2% normal swine serum in PBS, and the deparaffinized tissues were incubated overnight at 4°C after pretreatment by microwaving in 0.1 M citrate buffer solution for 20 min at 750 W for antigen retrieval. After washing in PBS, slides were incubated with biotin-labeled antimouse secondary antibodies (Vector Laboratories Inc., Burlingame, CA), then washed in PBS, and incubated with peroxidase-labeled streptavidin (DAKO). The reaction products were visualized by immersing the slides in freshly prepared diaminobenzidine (Dojindo, Kumamoto, Japan).

**Measurement of MVD.** Small blood vessels were visualized by staining endothelial cells with CD31 antibody. Two or three high-power fields were identified on each slide, and the MVDs were calculated as the mean number of vessels in a \( \times 200 \) field. Areas of inflammation, sclerotic tumor, and adjacent benign tissue were excluded when calculating the MVD.

**Calculation of Image Analysis.** The entire surface area of microvessels and the hypoxic ratio were determined with a computed image analyzer, KS 300 system version 2.00 (Karl Zeiss Vision K.K., Jena, Germany). Immunohistochemically stained sections of whole biopsy specimens at \( \times 100 \) were captured as images visualized on a computer screen. All of the microvessels that were immunohistochemically stained with CD31 antigen on each biopsy specimen were traced on an outline of the tumor tissue in the specimen, but normal laryngeal tissue, ulcerated tissue, and stromal tissue were excluded from the

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**Table 1** Clinicopathological features of 55 laryngeal carcinoma patients

<table>
<thead>
<tr>
<th>Variables</th>
<th>No. of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no. of patients</td>
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</tr>
<tr>
<td>Gender</td>
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<tr>
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<td>50</td>
</tr>
<tr>
<td>Female</td>
<td>5</td>
</tr>
<tr>
<td>Age</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>65.0 yr</td>
</tr>
<tr>
<td>Range</td>
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<td>Tumor location</td>
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<tr>
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<tr>
<td>Glottic</td>
<td>40</td>
</tr>
<tr>
<td>Subglottic</td>
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</tr>
<tr>
<td>T factor</td>
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<tr>
<td>Tis ( +T_1 )</td>
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<tr>
<td>T(_2)</td>
<td>24</td>
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<tr>
<td>N factor</td>
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<tr>
<td>( N_0 )</td>
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</tr>
<tr>
<td>( \geq N_2 )</td>
<td>6</td>
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<td>Well diff. SCC</td>
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</tr>
<tr>
<td>Moderately diff. SCC</td>
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<tr>
<td>Poorly diff. SCC</td>
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<tr>
<td>Undiff. ca.</td>
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</tr>
<tr>
<td>Verrucous ca.</td>
<td>1</td>
</tr>
<tr>
<td>C.I.S.</td>
<td>5</td>
</tr>
</tbody>
</table>

\( ^a \) diff., differentiated; ca., carcinoma; SCC, squamous cell ca.; C.I.S., ca. in situ.
The TP:TA ratio and TN:TA ratio are measured in units of vessels/mm². Whereas the TN:TA ratio is calculated as the ratio of total tumor tissue area (TA) divided by the total number of microvessels (TN), the TP:TA ratio is calculated as the ratio of the total number of microvessels (TP) divided by the total tumor tissue area (TA). These calculations were performed in each of the 55 cases. The TP:TA ratio was most significantly associated with radiosensitivity in the univariate analysis.

**RESULTS**

We assessed the clinicopathological characteristics and radiosensitivity. Of the 55 patients, 39 patients (70.9%) underwent 66 or 70 Gy radiotherapy, and the other 16 patients received 60 Gy or less. As shown in Table 2, there were no significant associations between radiosensitivity and the clinicopathological features, such as gender, age, tumor location, T factor, N factor, histological differentiation, or total radiation dose (P = 0.630, 0.105, 0.154, 0.626, 0.442, 0.567, and 0.560, respectively). Because none of the variables listed in Table 2 were significantly associated with radiosensitivity in the univariate analysis, we have not included a multivariate analysis.

The microvessels in the whole biopsy specimens from the 55 patients were stained with CD31 antibody. Total area, total microvessel number, total perimeter, and hypoxic area were measured in each biopsy specimen by using computed image analyzer, KS 300 system. MVD ranged from 11 to 109 vessels/field (mean ± SD, 41.6 ± 19.3 vessels/field), the TN:TA ratio ranged from 5.88 to 306.5 vessels/mm² (mean ± SD, 58.8 ± 54.7 vessels/mm²); the hypoxic ratio ranged from 0.36 to 86.4% (mean ± SD, 32.1 ± 22.2%); and the TP:TA ratio ranged from 0.53 to 13.95 mm/mm² (mean ± SD, 3.84 ± 2.98 mm²). The correlations between MVD, TN:TA ratio, TP:TA ratio or hypoxic ratio, and radiosensitivity are shown in Table 3.

Fig. 2 compares MVD, the TN:TA ratio, the TP:TA ratio, and the hypoxic ratio in both the radioresistant and radiosensitive cases. Mann-Whitney’s U test analysis revealed significant differences in each of the factors between the radiosensitive cases and the resistant cases (P = 0.002, 0.0004, <0.0001, and 0.004, respectively). The TP:TA ratio was most significantly associated with radiosensitivity. Kaplan-Meier analysis was performed for patients with a high and low MVD, TN:TA ratio, TP:TA ratio, or hypoxic ratio (Fig. 3). To assess the predictive value of the each factor (MVD, TN:TA ratio, TP:TA ratio, and hypoxic ratio) for radiosensitivity, all of the patients were divided into a high and a low group by using the median as the cutoff value. The LRC rate 3 years after treatment was 88.0% for those with a high MVD, but only 46.7% in those with a low MVD, 88.8% in those with a high TN:TA ratio, 34.4% in those with a low TN:TA ratio, 92.9% in those with a high TP:TA ratio, 37.0% in those with a low TP:TA ratio, 84.6% in those...
with a low hypoxic ratio, and 48.3% in those with a high hypoxic ratio. A significant association was found between the following factors: MVD, TP:TA ratio, and hypoxic ratio, and LRC (P = 0.004, 0.001, <0.0001, and = 0.011, respectively), but the strongest associated factor with LRC was the TP:TA ratio.

**DISCUSSION**

Biological factors that predict tumor radiosensitivity in *in vitro* and *in vivo* studies fall into three categories: (a) cell proliferation; (b) apoptosis; and (c) oxygen effect (10). We recently assessed the Ki-67 labeling and epidermal growth factor labeling index for cell proliferation, the apoptotic index for apoptosis, and the MVD for oxygen effect in 31 patients with laryngeal carcinoma who had undergone radical radiotherapy, and we found that MVD is a potentially useful factor for predicting radiosensitivity by multivariate analysis (7).

Oxygen status in tissue has been shown to be a significant, important factor that determines radiosensitivity both *in vitro* and in animal models (5, 6). In clinical specimens, Hockel et al. (12) and Brizel et al. (13) reported oxygen status measured by oxygen probes to be highly correlated with radiosensitivity in cervical carcinoma of uterus and in head and neck carcinoma. On the basis of *in vivo* observations of vascular geometry and blood flow in tumor microcirculation, oxygen delivery to tumor tissues appears to rely on a network of microvessels. In fact, Hulka et al. (8) and Secomb et al. (9) reported that MVD correlates well with blood flow in breast tumors. We, therefore, introduced tumor MVD in early-stage laryngeal carcinomas as a representation of O₂ status. Although we counted the number of

![Graphs showing MVD, TN:TA ratio, TP:TA ratio, and hypoxic ratio](image)

*Fig. 2* Mann-Whitney’s U test analysis of the relationships between radiosensitivity and MVD (*Microvessel Density*), TN:TA ratio (the ratio of total microvessel number to total tumor area), TP:TA ratio (the ratio of total microvessel perimeter to total tumor area), or hypoxic ratio in 55 laryngeal carcinomas. P = 0.002, 0.0004, <0.0001, and 0.004, respectively.

| Table 2  Relationship between clinicopathological features and radiosensitivity in 55 laryngeal carcinomas |
|-----------------|---------------|--------|--------|
| Variables          | Score | H.R.* | P     |
| Gender            |       |       |       |
| Male              | 0     | 0.604 |       |
| Female            | 1     | (0.078–4.697)* | 0.630 |
| Age               |       |       |       |
| <65 yr            | 0     | 1.011 |       |
| ≥65 yr            | 1     | (0.193–1.297) | 0.105 |
| Tumor location    |       |       |       |
| Supraglottic      | 0     | 0.500 |       |
| Glottic           | 1     | (0.193–1.297) | 0.154 |
| T factor          |       |       |       |
| Tis+T₁            | 0     | 0.799 |       |
| T₂               | 1     | (0.324–1.969) | 0.626 |
| N factor          |       |       |       |
| N₀                | 0     | 2.206 |       |
| ≥N₁              | 1     | (0.293–16.580) | 0.442 |
| Histological differentiation |   |       |       |
| W/D+M/D+Verrucous | 0     |       |       |
| P/D+Undiff.       | 1     | 1.806 |       |
| C.I.S.            | 2     | (0.239–13.629) | 0.567 |
| Radiation dose    |       |       |       |
| ≤60 Gy           | 0     | 1.321 |       |
| >60 Gy           | 1     | (0.518–3.372) | 0.560 |

*P*, hazard ratio; W/D, well differentiated squamous cell ca.; M/D, moderately differentiated squamous cell ca.; ca., carcinoma; P/D, poorly differentiated squamous cell ca.; C.I.S., *ca. in situ*; Undiff., undifferentiated.

*Numbers in parentheses, 95% confidence interval.*
microvessels in the previous study, the shape and size of each of the microvessels and of the biopsy specimens was not taken into consideration when measuring MVD at the hot spot. In addition to these sample variations, intra- and interobserver bias may occur and affect the objectivity of the data. To overcome these limitations in the previous study, we collected larger numbers of laryngeal carcinoma cases and used the computer image analyzer to measure the TN:TA ratio, the TP:TA ratio calculated from total microvessel numbers or perimeter divided by total tumor area, and the hypoxic ratio in tumor biopsy specimens after appropriate immunohistochemical staining of microvessels with antibody against CD31. We compared the reliability of these methods: MVD, TN:TA ratio, TP:TA ratio, and hypoxic ratio, as a means of predicting radiosensitivity and found that every parameter correlated significantly in statistical analysis. The present results objectively confirm the finding in our previous study that tumor oxygenation represented by MVD is a strong predictor of radiosensitivity in the early-stage laryngeal carcinomas. One of these parameters, the TP:TA ratio, has been shown to be the best predictive parameter for the radiosensitivity of laryngeal carcinomas. These findings combined with previous results indicate that the radiosensitivity of T1 and T2 stage small carcinomas is mainly affected not by cancer cell biology, such as genetic mutations, apoptosis, or tumor proliferative activity, but by an environmental factor surrounding the tumor cells: oxygen status.

Microvessel heterogeneity in tumors is a crucial issue in this study. More variation would be expected with smaller specimens, and this might be predicted to affect the mean MVD and hypoxic ratio more than the TN:TA ratio or the TP:TA ratios, which normalized to the total tissue that is available. Indeed, these last two parameters yielded more significant differences. Because all of the tumors used in the present study were small, i.e., 15 mm in diameter at most, and the biopsy specimens were 2–5 mm, there might be very little microvessel heterogeneity in such small tumors. Microvessels in biopsy specimens should represent the microvessels of T1 and T2 stage laryngeal carcinomas. In fact, we measured microvessel heterogeneity in both surgically resected T2 tumors and corresponding biopsy specimens in five cases and found that there was no significant difference between them (data not shown).

There is controversy concerning the choice of treatment, radiation therapy or surgical therapy, for T3 stage laryngeal carcinomas. If the results of our image analysis are applicable to T3 stage laryngeal carcinoma, the TP:TA ratio is more effective for selection of therapeutic method. However, the surface of larger tumors, such as in T3 cases, is often ulcerated, which may affect the number of microvessels in biopsy specimens. In fact,
there was one radioresistant tumor with an exceptionally high MVD, high TN:TA ratio, high TP:TA ratio, and low hypoxic ratio in the present study, and the specimen contained granulation tissue obtained from the ulcer in the tumor. Accordingly, ulceration should also be avoided in laryngeal biopsy specimens to accurately evaluate the TP:TA ratio of the tumor. The problem of sampling errors in single biopsy specimens of in vivo carcinoma may detract from our conclusion. Moreover, the median value was used as the cutoff value in this study, and there is still considerable overlap of the ranges of these parameters between radioresistant and sensitive tumors. Thus, it might be premature to state that a determination in an individual patient can be used to select radiation or surgery. This would be a reasonable question for a prospective study, in which many of these lesions would be amenable to either definitive radiation or surgical therapy.

Suboptimal radiation doses were administered in some cases in this study. In the recent Radiation Therapy Oncology Group (RTOG) Phase III trial, total radiation dose and dose/fraction were shown to be significantly more efficacious for local control (14). Higher total doses, to $\geq$72 Gy, or a hyperfractionated regime may be required for the more hypoxic tumors.

Prediction of radiosensitivity in the early-stage laryngeal carcinoma before radiotherapy is especially important from a clinical standpoint, because radiotherapy is the treatment of choice as a means of preserving patients’ phonation in T1 and T2 stage laryngeal carcinomas. Salvage laryngectomy after full-dose radiation in radioresistant cases is often followed by complications of salvage laryngectomy, such as pharyngo-cutaneous fistula and prolonged hospital stays. Greisen et al. (15) and Crellin et al. (16) reported fistula formation rates after salvage total laryngectomy of 11.5% and 26.8%, respectively. In our present study, complications after salvage surgery occurred in two (25.0%) of the eight patients who underwent total laryngectomy, and the average duration of nasal feeding in the eight patients reached 103.1 days.

Prediction of radiosensitivity in the early-stage laryngeal carcinoma on the basis of the TP:TA ratio can be an efficient means of avoiding ineffective radiation and complications.

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