Cyclooxygenase 2 Promoter–Based Replication-Selective Adenoviral Vector for Hypopharyngeal Cancer

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Objective: To explore the potential clinical application of the oncolytic activity of cyclooxygenase 2 (COX-2) promoter–based, conditional, replication-selective adenovirus vector for hypopharyngeal squamous cell carcinoma.

Design: In vivo study and retrospective study.

Setting: Kobe University Hospital, Kobe, Japan.

Subjects: Expression of COX-2 in hypopharyngeal cancers treated at Kobe University Hospital was immunohistochemically investigated. In addition, nude mice bearing human hypopharyngeal cancer cells (H891) were used to analyze oncolytic activity of a conditional replication-selective adenovirus vector in which the expression of E1a, required for viral replication, is controlled by the COX-2 promoter Ad-COX2-E1a.

Results: In vivo assays showed significant growth suppression in the murine hypopharyngeal model. Cyclooxygenase 2 expression was observed in 75.3% of hypopharyngeal cancers, especially in differentiated tumor cells (P=.001; r=0.433).

Conclusion: In this study, we demonstrated the potential of oncolytic therapy using the COX-2–promoter based, conditional, replication-selective adenovirus for COX-2–expressing hypopharyngeal squamous cell carcinomas.


Adenoviruses have been widely used as convenient and safe vectors for transferring therapeutic genes into target cancer cells. Basically, genes such as E1a and E1b, which are essential for the replication of the adenoviruses, are artificially removed from the adenoviral vectors to eliminate proliferation of the vectors. Thus these vectors, restriction-defective adenoviral vectors, pose no danger of uncontrolled proliferation. However, they have limited efficacy of treatment because of the limited number of tumor cells to which the therapeutic gene can be delivered.

To overcome this limitation, recent advances in molecular biology have made it possible to genetically reengineer viruses to target tumor cells selectively. These viruses are known as selectively replicative viruses and are designed for limited ability to replicate themselves only in the targeted tumor cells but not in normal tissues.1 Tumor cell killing is achieved not by the genes delivered by the vectors but by the oncolysis induced by the replicated viruses because of their original characteristics as adenoviruses.2 Amplified viral vectors also spread to the adjacent tumor cells and kill these cells in the same manner.3

Cyclooxygenase 2 (COX-2), which is primarily responsible for prostaglandins produced at inflammatory sites, is virtually undetectable in most tissues under physiologic conditions but is upregulated in various cancers including head and neck cancers.4 To take advantage of this tumor-specific expression, we have recently generated the COX-2 promoter–based replication-selective adenoviral vector, Ad-COX2-E1a, and have demonstrated its antitumor effect against COX-2–expressing hypopharyngeal squamous cell carcinoma in an in vitro study.3

To further explore the potential for clinical application of Ad-COX2-E1a for the treatment of head and neck cancer, the present study investigated COX-2 expression in hypopharyngeal cancers using immunohistochemical techniques. The oncolytic activity of Ad-COX2-E1a in vivo was also tested using nude mice bearing human hypopharyngeal cancer cells (H891).
Tumor tissues obtained at biopsy were fixed in formalin and paraffin embedded according to standard procedures. Tissue sections with a thickness of 4 µm were deparaffinized in xylene, rehydrated, treated with 3% hydrogen peroxide in methanol for 5 minutes to block endogenous peroxidase activity, and treated with Proteinase K (DakoCytomation, Carpentaria, California) for 10 minutes for proteolytic digestion to improve the accessibility of antibodies.

Sections were incubated with COX-2 Polyclonal Antibody (Cayman Chemical Co, Ann Arbor, Michigan) for 30 minutes, then with antirabbit IgG for 30 minutes, and finally treated with Vectastain Elite ABC peroxidase kit (Vector Laboratories Inc, Burlingame, California) and visualized with diamino benzidine.
The associations between clinicopathological features and COX-2 expression are listed in Table 2. No significant association was observed between COX-2 expression and age, sex, stage, pathologic T stage, or pathologic N stage. Figure 4 shows survival curves of the patients with hypopharyngeal cancer in relation to COX-2 expression. The 5-year survival rates of patients with and without COX-2 expression were 54% and 43%, respectively (P = .21).

Figure 1. Scoring of staining intensity for cyclooxygenase 2 (COX-2). The specimens in the top row (A-D) were treated with immunohistochemical staining for COX-2; those in the bottom row (E-H), hematoxylin-eosin (original magnification ×40). Staining intensity was scored as 0 for negative (A and E), 1 for weak (B and F), 2 for medium (C and G), and 3 for strong (D and H).

Figure 2. Oncolytic activity of As-COX2-E1a in vivo. The H891 cells (1 × 10⁶) were subcutaneously injected into the backs of male nude mice. When tumors with a 5- to 6-mm diameter had developed, mice were given phosphate-buffered saline, Ad-CMV-LacZ, or Ad-COX2-E1a. Growth of tumors treated with Ad-CMV-LacZ (B) was significantly greater than that of those treated with Ad-COX2-E1a (C) 14 days after injection. The scale lines in panels A and C represent millimeters.

Figure 3. Inhibition of human head and neck squamous cell carcinoma and 293 cell lines in vitro. Growth of human hypopharyngeal cancer was significantly inhibited by Ad-COX2-E1a compared with tumors treated with Ad-CMV-LacZ or phosphate-buffered saline (PBS) at 14 days after injection.

Figure 4. Survival curves of the patients with hypopharyngeal cancer in relation to COX-2 expression. The 5-year survival rates of patients with and without COX-2 expression were 54% and 43%, respectively (P = .21).

COMMENT

Cyclooxygenase 2, which is primarily responsible for prostaglandins produced at inflammatory sites, is virtually undetectable in most tissues under physiologic conditions. In contrast, recent studies have demonstrated that COX-2 is expressed in several cancer tissues, including head and neck cancers, and may be important for carcinogenesis. Taking advantage of this tumor-specific expression of COX-2, our research group has recently generated the COX-2 promoter–based replication-selective adenoviral vector Ad-COX2-E1a and have demonstrated its antitumor effect against COX-2–
expressing hypopharyngeal squamous cell carcinoma in an in vitro study.\textsuperscript{3}

Herein, we show that Ad-COX-2-E1a also significantly inhibited the growth of COX-2–expressing tumors without serious adverse effects in an animal model. In addition, immunohistochemical analysis demonstrated that 75.3% of the hypopharyngeal cancers expressed COX-2, which suggests that about three-fourths of patients with hypopharyngeal cancer are potential candidates for this treatment.

Tumor cell differentiation was significantly related to COX-2 expression, which was upregulated in well-differentiated tumors, suggesting that its expression may be involved in the pathogenesis or growth of well-differentiated tumor cells. Alternatively, COX-2 overexpression in these tumors may be a consequence of squamous differentiation in an abnormal setting. In this regard, it should be noted that COX-2 expression becomes weaker when hypopharyngeal cancer progresses to a more aggressive phenotype, thus becoming less differentiated. Indeed, while statistically not significant, the 5-year survival rate of patients without COX-2 expression was worse than that of the patients with COX-2 expression. Cyclooxygenase 2 expression may therefore indicate a relatively favorable condition, even though the prognosis of patients with COX-2–expressing hypopharyngeal cancer remains unsatisfactory.

In terms of therapeutic strategy, the effects of chemotherapy or radiotherapy are generally less favorable for well-differentiated tumors.\textsuperscript{14,15} On the other hand, Ad-COX2-E1a is more effective for COX-2–expressing tumors, as evident in this and our group’s previous study.\textsuperscript{3} Since more than half of the differentiated tumors in this study expressed COX-2, Ad-COX2-E1a may compensate for the chemoresistance and/or radioresistance of well-differentiated cancers.

Adenoviral therapy in combination with chemotherapy has shown promising results in various preclinical models and clinical trials.\textsuperscript{16} In particular, the treatment regimen of intratumoral dl1520 injection in combination with cisplatin and 5-fluorouracil for patients with recurrent head and neck cancer has already entered phase 2 trials and shown promising results.\textsuperscript{17,18} As reported by many investigators, direct intratumoral injection is more efficient than other delivery methods, eg, intravenous, intra-arterial, or intraperitoneal injection.\textsuperscript{19-23} Since the head and neck areas are easy to approach, patients with head and neck cancer are thought to be some of the most suitable candidates for adenoviral therapy.

Recently, many studies have reported that selective COX-2 inhibitors are useful in prevention or treating various neoplasms, including head and neck cancer.\textsuperscript{24-27} The

### Table 1. Intensity of Staining for Cyclooxygenase 2

<table>
<thead>
<tr>
<th>Tumor Cell Characteristic</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Well differentiated</td>
<td>4</td>
<td>18</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Moderately differentiated</td>
<td>12</td>
<td>24</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>7</td>
<td>2</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Scored as 0 for negative, 1 for weak, 2 for medium, and 3 for strong.

### Table 2. Cyclooxygenase 2 (COX-2) Expression According to the Clinical Characteristics of the Patients\textsuperscript{a}

<table>
<thead>
<tr>
<th>Clinical Features\textsuperscript{b}</th>
<th>Patients</th>
<th>COX-2 Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative (n = 23)</td>
<td>Positive (n = 70)</td>
</tr>
<tr>
<td>Sex</td>
<td>85</td>
<td>19</td>
</tr>
<tr>
<td>Male</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Female</td>
<td>46</td>
<td>14</td>
</tr>
<tr>
<td>Age, y</td>
<td>47</td>
<td>9</td>
</tr>
<tr>
<td>&lt;65</td>
<td>18</td>
<td>5</td>
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<tr>
<td>\geq 65</td>
<td>75</td>
<td>18</td>
</tr>
<tr>
<td>Stage</td>
<td>43</td>
<td>11</td>
</tr>
<tr>
<td>II and III</td>
<td>50</td>
<td>12</td>
</tr>
<tr>
<td>IV</td>
<td>20</td>
<td>7</td>
</tr>
<tr>
<td>pT</td>
<td>73</td>
<td>16</td>
</tr>
</tbody>
</table>

Abbreviations: pN, pathologic N stage; pT, pathologic T stage.

\textsuperscript{a} Unless otherwise indicated, data are reported as number of patients.

\textsuperscript{b} No statistically significant difference was found for any category.

### Figure 4. Survival of the patients with hypopharyngeal cancer. The top line shows the survival curve of the patients with hypopharyngeal cancer without cyclooxygenase 2 (COX-2) expression. The bottom line shows the survival curve of the patients with hypopharyngeal cancer with COX-2 expression. Although statistical analysis did not show a significant difference, the survival rate of patients with hypopharyngeal cancer expressing CDX-2 was better than that of those whose cancer was without COX-2 expression.
effects are related to suppression of cell proliferation and induction of apoptosis, and the expression of COX-2 itself is not suppressed by selective COX-2 inhibitors.\textsuperscript{24}

Because the effect of Ad-COX2-E1a depends on the expression of COX-2 itself, the combination therapy of Ad-COX2-E1a and selective COX-2-inhibitors may lead to the synergetic effects in COX-2–expressing head and neck cancers.

Although viral introduction as part of adenoviral therapy is highly efficient, injury to normal organs must be avoided. In this connection, newer generations of adenoviral vector have recently been developed to further restrict viral replication to tumor cells. Further studies would make the implementation of chemoradiotherapy with selectively replicative viruses a reality.

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Acquisition of data: Nakagawa, Tanaka, Hamada, and Tsukuda.

Analysis and interpretation of data: Nakagawa, Hayashi, Tsukuda, and Nibu.

Drafting of the manuscript: Nakagawa and Nibu.

Critical revision of the manuscript for important intellectual content: Tanaka, Shirakawa, Gotoh, Hayashi, Hamada, Tsukuda, and Nibu.

Obtained funding: Nibu.

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Study supervision: Hayashi, Hamada, and Nibu.

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