Expression of Cox-2 protein in radioresistant laryngeal cancer

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Background: Radiotherapy is the principal modality used to treat early stage laryngeal cancer. Unfortunately treatment failures occur in 10–25% of patients. Subsequent salvage surgery is technically more difficult, with increased complication and failure rates. The ability to predict or prevent radioresistance would improve the poor survival associated with this disease. Cox-2 is an inducible enzyme involved with prostaglandin synthesis. We investigated a potential role for Cox-2 in predicting radioresistance in laryngeal cancer.

Patients and methods: Using immunohistochemical techniques we examined the expression of Cox-2 protein in 122 pre-treatment laryngeal biopsies. All tumours were treated with single modality radiotherapy (curative intent). The group comprised of 61 radioresistant and 61 radiosensitive tumours matched for T stage, laryngeal subsite, gender and smoking history.

Results: Cox-2 expression was detected in 41 of 61 (67%) biopsy samples from patients with radioresistant tumours and 25 of 61 (41%) radiosensitive tumours. Overexpression was significantly associated with radioresistant tumours ($P = 0.004$). Cox-2 has a 67% accuracy in predicting radiotherapy failure.

Conclusion: Cox-2 may have prognostic value in predicting response to radiotherapy. Cox-2 inhibitors such as NS-398 have been shown to enhance the effects of radiotherapy. We suggest that their use may be beneficial in patients who are destined to fail radiotherapy.

Key words: Cox-2; laryngeal cancer; radioresistance

Introduction

Laryngeal cancer represents the largest subgroup of head and neck cancers, which comprise a diverse group of tumours arising from the epithelium of the upper aerodigestive tract, paranasal sinuses, salivary and thyroid glands [1]. In 2000 the global incidence of laryngeal cancer was 142 000 cases [2]. Treatment options comprise radiotherapy, surgery, chemotherapy or a combination of modalities [3]. Despite refinement of multimodal therapies over the last 20 years, 5-year survival figures remain poor. Five-year survival rates of 40% are quoted and these figures have remained static since the mid-1980s [2, 4].

Radiotherapy used as a single treatment modality can be an effective cure for early stage (T1 and T2) laryngeal tumours. Radiotherapy treatment has the advantage over a total laryngectomy in that it spares functional anatomy and the patient is able to speak and swallow normally following therapy. The loss of such abilities has a significant impact on the quality of a patient’s life. The subsequent impaired ability to communicate and the disfiguring surgery also have a detrimental psychological impact. Unfortunately radiotherapy treatment failures do occur. Approximately 10% of patients with stage I disease [5] and 25% of patients with stage II disease [6] do not respond to radiotherapy. These observations show that the TNM (tumour–node–metastasis) system, although widely used as the basis for patient cancer management, cannot however predict an individual tumour response to radiotherapy [7].

If a patient fails radiotherapy, a total laryngectomy is the main treatment option that may offer a cure. The loss of the larynx has a significant psychological impact upon the patient, especially one who has undergone a failed course of radiotherapy. Operating in a previously irradiated field results in increased surgical failure and complication rates [8]. More importantly, definitive cancer cure is delayed by the course of radiotherapy. Due to this delay tumour progression may have occurred, adversely affecting patient prognosis still further.

The prostaglandin system is composed of two distinct isoenzymes: cyclooxygenase 1 and 2. These enzymes form part of the prostaglandin synthetase complex of enzymes, which play a key role in the conversion of arachidonic acid into prostaglandin G2 and H2. Prostaglandin H2 is then transformed into individual prostaglandins (PGD2, PGE2, PGF2α, PGI2, TXA2) by tissue-specific components of the synthetase complex. These individual components can then exert their effects on angiogenesis, apoptosis and immune surveillance. Recently, Cox-2 protein overexpression has been reported to correlate with decreased survival in patients with cervical cancer after treatment with radiotherapy [9]. Cox-2 is an inducible enzyme not normally present in normal tissue in contrast to Cox-1, which is expressed at a constant level throughout the cell cycle by almost all tissues [10]. Cox-2 can be induced by a variety of stimuli including cytokines [11], growth factors [12] and oncogenes [10]. Prostaglandins regulated by...
Cox-2 are believed to be important in the pathogenesis of cancer due to their effects on apoptosis, angiogenesis, cell proliferation and immune surveillance [10, 13]. The precise mechanistic role for Cox-2 in tumorigenesis continues to be evaluated; however, its overexpression is believed to play an important role [10]. In support of this, Oshim et al. have demonstrated that ‘knocking out’ the Cox-2 gene leads to a marked reduction in intestinal polyps, in a mouse model of familial adenomatous polyposis [14].

Cox-2 overexpression has also been implicated in tumour response to radiotherapy. Tsujii and Dubois demonstrated that cell lines which overexpress Cox-2 were resistant to apoptosis, an important pathway of cell death induced by ionising radiation [15]. Pyo et al. demonstrated that the Cox-2 inhibitor, NS-398, enhances the effect of radiotherapy in vitro and in vivo on human cells that overexpress Cox-2 [16]. These authors reported that the radiation-enhancing effects of NS-398 did not occur in cells deficient in Cox-2 expression and concluded that Cox-2 overexpression was essential for the effects of NS-398. On the basis of these observations we investigated the possible relationship between Cox-2 protein expression and treatment failure in laryngeal cancer treated with radiotherapy.

Patients and methods

Local Research Ethics Committee approval for obtaining data and archival biopsy material for the study was obtained. Patients diagnosed with laryngeal carcinoma and treated with single modality radiotherapy with curative intent (either 55 Gy in 20 fractions or 60 Gy in 25 fractions) were identified from databases held in ENT departments in England. Patients were identified as having radioresistant or radiosensitive tumours depending upon their response to radiotherapy. In order to reduce confounding variables, the radioresistant and radiosensitive groups were matched with regards to T stage, laryngeal subsite and smoking history. Tumours were staged according to the TNM classification [17] and all were clinically nodal negative (N0) and metastatic negative (M0) at the time of treatment.

The radioresistant group consisted of 61 patients: 43 stage T1 and 18 stage T2 laryngeal squamous cell carcinomas. The criteria for a radioresistant tumour were: (a) the radiotherapy had to be given as a single modality treatment with curative intent for a biopsy-proven squamous cell carcinoma of the larynx; (b) biopsy-proven recurrent squamous cell carcinoma, the recurrence occurring at the original anatomical site, within 12 months of finishing a course of radiotherapy.

The radiosensitive group of tumours consisted of 61 patients: 43 stage T1 and 18 stage T2 squamous cell carcinomas of the larynx. The criteria for a radiosensitive tumour were: (a) the radiotherapy had to be given as a single modality treatment with curative intent for a biopsy-proven squamous cell carcinoma of the larynx; (b) biopsy-proven recurrent squamous cell carcinoma, the recurrence occurring at the original anatomical site, within 12 months of finishing a course of radiotherapy.

Tissue sections (4 μm) were cut from pre-treatment archival tissue blocks of the radioresistant and radiosensitive tumours. Immunohistochemistry as previously described was used to detect Cox-2 on the tissue sections [18]. In brief, antigen retrieval was performed using pressurised heat retrieval. The primary antibody (100 μl, anti-Cox-2 (BD Biosciences, USA; Cat no. 610203 clone 33), at a dilution of 1:50 (diluted in 0.2% casein) was added to each tissue section and incubated at room temperature for 2 h. A negative control was included using 100 μl of 0.2% casein instead of the primary antibody. The Duet kit (DAKO, Denmark) was used as the secondary detection system and 3′,3′-diaminobenzidine tetrachloride as the chromogen. Two assessors scored the anti-Cox-2 staining independently with the radiotherapy treatment outcome blinded to the assessor. A simple scoring system analysing only tumour cells was used to interpret the staining patterns [19]. Sections were regarded as positive if >5% of the tumour cells stained. Sections with ≤5% of the tumour staining were considered negative. In order to reduce sampling error the whole biopsy section for each tumour was analysed.

Statistics

Chi-squared statistical analysis using SPSS version 11.5 (SPSS Inc, Chicago, USA) was used throughout. All P values quoted are for two-sided significance, between the radioresistant and radiosensitive groups. Values <0.05 were considered significant. Marker sensitivity and specificity were calculated as previously described [20].

Results

One hundred and twenty-two patients with laryngeal squamous carcinoma were treated with single modality radiotherapy given with curative intent. No patient had a previous diagnosis of cancer and there was no evidence of regional or distant tumour metastasis. The two patient groups were matched as closely as possible; the clinicopathological details are shown in Table 1. There was no significant difference in tumour differentiation (P = 0.543) or gender (P = 0.610) between the two groups.

Cox-2 staining, when present, was evident in the cytoplasm of tumour cells (Figures 1 and 2). Preservation of Cox-2 antigen was evident in Cox-2-negative tumours as occasional stromal cells were positive in the tissue sections. This served as an internal positive control in Cox-2-negative tumours. Ferrandina et al. has reported a similar finding when investigating Cox-2 expression in squamous cell carcinoma of the cervix [21].

Cox-2 overexpression was significantly associated with radioresistant tumours (P = 0.004). In the radioresistant group, 41 of 61
(67%) tumours were positive for Cox-2 overexpression. In comparison only 25 of 61 (41%) radiosensitive tumours were positive. There was no significant association of Cox-2 overexpression with T stage ($P = 0.08$), degree of tumour differentiation ($P = 0.181$) or laryngeal glottic or supraglottic subite ($P = 0.757$). Cox-2 expression was also not related to time of recurrence in the radioresistant group ($P = 0.126$).

If Cox-2 is used as a predictive marker for radiotherapy outcome in early stage laryngeal cancer it has an accuracy of 67% (Table 2).

### Discussion

Unfortunately, no universal definition of a radioresistant tumour exists [22]. This poses an obvious problem when evaluating published research in this area [7]. In this series a strict definition of radioresistance has been used. The recurrences had to occur at the original anatomical site following a course of radical radiotherapy. The recurrence had to be of a similar histology and occur within 12 months of finishing the course of radiotherapy. It is hoped that such a definition will exclude second primary tumours, that are common in the head and neck region and occur at a constant rate of $\sim 7\%$ per year [23]. If these second primary tumours were not excluded they would be interpreted as a radiotherapy recurrence. Smith and Haffty [7] reviewed recent studies evaluating molecular markers including p53, angiogenesis-related markers, cyclin D1, epidermal growth factor receptor, DNA ploidy and cell kinetic markers in radioresistant head and neck cancers. They concluded that future studies evaluating larger patient populations with a narrower range of stages and sites of disease that are treated in a uniform fashion should limit the heterogeneity of published results. Studies specifically investigating radioresistant early stage laryngeal cancer have reported mixed results with regards to the above markers [24]. This is probably due to the small number of cases examined (<20) in each study. As a consequence, molecular markers of radioresistance have yet to contribute to the clinical decision process.

Head and neck cancers comprise a heterogeneous group of tumours arising from the upper aerodigestive tract, paranasal sinuses and glandular tissue. Each region has its own TNM staging system and differing response rates to radiotherapy [3]. We have limited this series to the larynx, which forms the largest subgroup of head and neck cancers. This is again to reduce confounding factors that may affect results from mixed tumour groups, as it is well documented that for a given T stage regional variation for treatment modality and outcome exists [3].

Using only pre-treatment tissue we have demonstrated that Cox-2 is overexpressed in laryngeal cancer. This is in agreement with previous observations of Cox-2 protein expression in head and neck cancer. Chan et al. reported that Cox-2 is expressed in 100% of tumours from a mixed group of 10 head and neck patients [24]. Ranelletti et al. reported Cox-2 overexpression in 31% (19 of 61) of tumours that comprised 18 stage I or II laryngeal cancers and 43 stage III or IV laryngeal cancers [25].

Our series represent a large collection of radioresistant tumours from one head and neck sub-site. It is the first study to document the overexpression of Cox-2 in such a group. We have found that 67% of radioresistant tumours overexpress Cox-2 in pre-treat-

### Table 2. Predictive value of Cox-2 as a marker of radiotherapy outcome in 122 patients with early stage laryngeal cancer

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<th>Cox-2 staining (n)</th>
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<td></td>
<td>Tumour recurrence (61 patients) (n)</td>
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<td>Positive (66)</td>
<td>True +ve (41)</td>
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<td>Negative (56)</td>
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ment biopsy samples. The radioresistant tumour group have also been matched for T stage, laryngeal subsite and smoking history to a group of radiosensitive tumours. We have shown that Cox-2 overexpression has a greater association with radioresistant tumours than radiosensitive tumours ($P = 0.004$).

The association of Cox-2 with the radioresistant tumours has two potentially important consequences. First, as this association is present in pre-treatment biopsy material it may be used as a prognostic marker predicting radiotherapy treatment failure with an accuracy of $67\%$. Cox-2-positive patients with early T1 or T2 laryngeal cancer could be offered partial laryngeal surgery as a first-line treatment instead of radiotherapy. This treatment option is widely used in the USA and is equally as effective as radiotherapy for early stage laryngeal tumours [3]. Consequently such patients will not require salvage surgery and will benefit from improved survival and quality of life as their larynx will be preserved and they will not have to receive unnecessary radiotherapy. Equally there will be no detrimental effect to the $20\%$ of patients with a false positive Cox-2 result who will be offered partial laryngeal surgery instead of radiotherapy. The accuracy of prediction may be increased if further markers can be identified which could be used in combination with Cox-2.

Second, the use of Cox-2 inhibitors may be able to enhance the effects of radiotherapy. Cells overexpressing Cox-2 have been shown to be resistant to apoptosis, an important mechanism of radiation-induced cell death [15]. The authors attributed this effect to increased levels of the important anti-apoptotic protein bcl-2, in epithelial cells, with forced overexpression of Cox-2. In the clinical setting, overexpression of Cox-2 has been associated with radiotherapy treatment failures in cervical cancer [9, 26, 27].

Inhibitors of Cox-2 have been shown to induce apoptosis in tumour cells [28, 29]. The Cox-2 inhibitor, NS-398, enhances the effect of radiotherapy, in vitro and in vivo, preferentially on human cell lines that overexpress Cox-2 [16]. Selective Cox-2 inhibitors, NS-398 and SC-236 [30], are non-toxic analgesic agents, which do not have the common gastrointestinal side of the commonly used non-selective Cox inhibitors such as aspirin [31]. It has also been shown that Cox-2 inhibitors do not affect the radiotherapy response of normal tissue in mice [32]. This is probably due to the lack of Cox-2 expression in normal tissue. This would allow the therapeutic ratio to be increased if Cox-2 inhibitors were used as a radiosensitiser for human cancers. By using Cox-2 inhibitors the clinician may be able to radiosensitise laryngeal cancer. This would result in fewer radiotherapy treatment failures. It would also spare the patient the functional loss and psychological impact associated with salvage surgery following treatment failure. Cox-2 inhibitors may also be beneficial to patients with advanced stage laryngeal cancer, who do not have the alternative treatment options that are available for early stage tumours. Advanced tumours are generally treated with combined surgery and radiotherapy in contrast to single modality treatment of early stage tumours [3].

Despite advances in radiotherapy treatment for laryngeal cancer, patient survival has not improved significantly over the last two decades [2]. The ability to predict radioresistant tumours would allow the clinician to use alternative therapies, aimed at achieving tumour control. Understanding the mechanism of tumour radioresistance will allow innovative therapies to be devised. Inhibitors of Cox-2 have the ability to enhance the effects of radiation. By using such inhibitors to radiosensitise tumours, enhanced radiotherapy cure rates may be attained. It is hoped that such strategies would improve the poor survival figures for head and neck cancer that have remained static since the 1980s.

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References