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Main Article

Changes in expression of p53, proliferating cell nuclear antigen and bcl-2 in recurrent laryngeal cancer after radiotherapy

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Abstract

The biological changes in recurrent laryngeal cancer following radiotherapy are not fully understood. The authors investigated differences in the expression of p53, proliferating cell nuclear antigen (PCNA) and bcl-2 in laryngeal cancer specimens before radiotherapy and in recurrent laryngeal cancer specimens following radiotherapy in the same patients. The authors investigated the expression of p53, PCNA and bcl-2 by immunohistochemical stain in 30 specimens from 15 patients with primary laryngeal cancer and recurrent laryngeal cancer after radiotherapy.

The expression of p53 protein was significantly different in laryngeal cancer before radiotherapy (4/15, 26.7 per cent) compared with recurrent laryngeal cancer after radiotherapy (8/15, 53.3 per cent) ($p < 0.05$). The PCNA index was also significantly different in laryngeal cancer specimens before radiotherapy (mean, 11.9 per cent) compared with recurrent laryngeal cancer after radiotherapy (mean, 18.0 per cent) ($p < 0.05$). However, there was no statistically significant alteration of bcl-2 expression in primary compared with recurrent laryngeal cancer. The expression of p53 and PCNA increased in recurrent laryngeal cancers after radiotherapy, compared with that in laryngeal cancers before radiotherapy. Recurrent laryngeal cancers arising following radiotherapy became biologically aggressive.

Key words: Laryngeal Neoplasms; P53 Genes; Proliferating Nuclear Cell Antigen; Bcl-2 Genes; Radiotherapy

Introduction

Radiotherapy plays an increasingly important role in the primary treatment of head and neck cancers such as early laryngeal cancer. Radiation has direct and indirect effects: radiation causes deoxyribonucleic acid (DNA)-double-strand breakage in cancer cell DNA, and it also creates HO free radicals that act on surrounding tissues.[1,2] Because of the DNA damage, these genetic alterations may lead to a more aggressive phenotype in locally recurrent laryngeal cancer following radiotherapy. Moreover, there is a possibility that the genetic changes may be related to resistance to radiotherapy.

This study was carried out to demonstrate molecular markers that could more accurately characterize the biological changes of recurrent laryngeal cancers following radiotherapy. Because the expression of p53, proliferating cell nuclear antigen (PCNA) and bcl-2 are important factors involved in tumour progression and the response of treatment, we chose to examine the expression of p53, PCNA and bcl-2 in primary and recurrent tumour specimens.

Material and methods

Subjects

The study group consisted of 15 patients treated by salvage total laryngectomy or supracricoid partial laryngectomy for persistent or recurrent laryngeal cancer following radiation therapy, between January 1995 and December 2002 at the Pusan National University Hospital. All patients with early glottic cancer (T_1 or T_2 N_0 M_0) and histopathologically confirmed squamous cell carcinoma of the glottis before radiation therapy were treated with radiation therapy alone with a curative intent. A median dose of 70.2 Gy was given over seven weeks with cobalt-60. The subjects were all male and their average age was 58.7 years. The average period from radiotherapy to recurrence was 14.8 months (range, one to 74 months).

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Immunohistochemistry

Immunohistochemical analysis of p53, PCNA and bcl-2 (Dako, Carpinteria CA, USA) was performed. Five-micrometre-thick sections of paraffin-embedded tumour tissues were used for immunohistochemical staining with the streptavadin-biotin peroxidase technique (Universal LSAB kit, Dako). The paraffin sections were deparaffinized using xylene and rehydrated using sequential concentrated alcohol. To enhance staining, the slides were subjected to microwave antigen retrieval (700 W, 2×5 min) in 0.01 M sodium citrate buffer (pH 6.0), and the endogenous hydrogen peroxidase activity was blocked by 3 per cent H_2O_2 for 10 min. To minimize nonspecific binding, the tissue of each slide was reacted with a protein-blocking agent (Dako) for 20 min. The specimens were incubated with primary antibodies at room temperature for one hour, washed in phosphate-buffered saline (PBS) three times, incubated with biotinylated anti-rabbit and anti-mouse immunoglobulin G for 30 min and then washed three times. The slides were treated with streptavadin buffer diluent for 20 min at room temperature and washed with PSB three times. The peroxidase reaction was visualized using 3-amino-9-ethylcarbazole (Vector laboratories, Burlingame CA, USA) for three to 10 min.

Determination of degree of stain

As a negative control, a section of each sample was processed with the first antibody omitted. A positive response was identified when distinct reddish brown reactions appeared in the nuclei of more than 10 per cent of the tumour cells in the p53 immunohistochemical stain. A positive response for PCNA was also identified when reddish brown stain reactions were observed in the nuclei of tumour cells. The percentage of nuclei stained, the PCNA index, was assessed by counting all tumour cells in 10 randomly selected high-power fields ($\times 400$). At least 500 cells were counted. The expression of bcl-2 was categorized as negative (no staining was seen in tumour cells) or positive (30 per cent or more of the cytoplasm of tumour cells were intensely stained).

Statistical analysis

Paired *t*-tests were conducted on the changes in expression of p53, PCNA and bcl-2 in tissues before and after radiation therapy; a p value of 0.05 or below was regarded as significant.

Results

Before radiation, four (26.7 per cent) out of the 15 cases showed positive p53 expression and 11 cases (73.3 per cent) showed negative expression. In the recurring cancers, however, the number of cases with positive p53 expression increased to eight (53.3 per cent) out of the 15 cases (Table I, Figure 1) ($p < 0.05$).

Before radiation, the PCNA index of cancer tissues was 11.9 per cent on average (0.2–0.8 per cent). However, the PCNA index in recurring cancer tissues increased to 18.0 per cent on average (7.0–39.6 per cent) (Table I, Figure 2) ($p < 0.05$). There were no statistically significant changes in bcl-2 expression in primary (7/15, 46.7 per cent) compared with recurrent laryngeal cancer (5/15, 33.3 per cent) (Table I) ($p > 0.05$).

Discussion

Biological changes in recurrent malignant cells following radiotherapy have not been well characterized to date. The possible mechanisms of recurrent cancer following radiation include: (1) existing cells with tolerance to radioactivity survive the radiation and recur; (2) radiation causes changes to the existing cancer, which recurs as another type of cancer; and (3) a new tumour develops. We do not know which mechanism causes recurrences. However, it is possible that malignant cells which persist or recur locally following radiotherapy possess additional genetic alterations compared with the primary, pre-radiotherapy tumour. Furthermore, because of the DNA-damaging effects of radiation, these genetic alterations may lead to more malignant phenotypes in locally recurrent cancer following radiotherapy, compared with the pre-radiotherapy tumour. Any genetic changes caused by failed radiotherapy may have profound implications on the response to further treatment, so determining pre-radiotherapy tumour characteristics has been less useful for predicting how patients will response to additional treatment. The alteration of the expression of p53, PCNA and bcl-2 in locally recurrent laryngeal cancers following radiotherapy may support this hypothesis.

Radiotherapy may have a deleterious effect on patients who are not cured by this treatment. Recurrent prostate tumours following radiotherapy may grow more rapidly than primary, untreated tumours.[3] Locally recurrent prostate tumours following radiotherapy are more poorly differentiated and have a more aggressive phenotype than the same tumours before radiotherapy.[4]

Mutations in the p53 gene are the most common genetic abnormality in all human cancers. This gene plays a critical role in growth inhibition, cellular response to genetic damage and entrance into the apoptotic pathway. Our results demonstrate a significant increase in p53 expression in recurrent laryngeal cancers following radiotherapy, compared with that in primary, pre-radiotherapy cancers ($p < 0.05$). This is consistent with the finding that recurrent prostate cancer following radiotherapy showed a higher rate of p53 mutation than did the prostate cancer before radiotherapy.[5] In addition, radiotherapy may induce p53 mutation in lung cancer.[6] The increased incidence of p53 alterations may reflect new genetic mutations and selective growth of cells containing a p53 mutation or p53 protein alteration in the absence of gene mutation. Recurrent laryngeal cancers following radiotherapy showed an increase of p53 expression, which suggests that p53 mutation may be related to sensitivity to radiotherapy. These results are consistent with the finding[7–9] that p53 expression correlates with resistance to radiation.

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TABLE I

PATIENT CHARACTERISTICS AND IMMUNOHISTOCHEMICAL RESULTS

Case	Sex/age (years)	Operation	Before radiation		After radiation	
			p53/bcl-2	PCNA index	p53/bcl-2	PCNA index
1	M/54	SCPL	−/−	0.2	−/+	7.0
2	M/66	SCPL	−/+	11.8	−/+	13.0
3	M/57	TL	−/−	5.2	+/−	10.0
4	M/45	SCPL	−/+	8.2	−/−	7.6
5	M/52	TL	−/+	10.2	−	14.4
6	M/55	SCPL	−/−	7.4	−/−	10.6
7	M/66	TL	+/−	20.2	+/−	39.6
8	M/77	TL	+/−	5.2	+/−	9.2
9	M/55	SCPL	−/+	13.2	−/+	10.2
10	M/54	SCPL	−/+	17.4	+/−	20.4
11	M/67	SCPL	−/+	16.2	−/+	9.2
12	M/64	TL	−/+	20.8	+/−	29.2
13	M/54	SCPL	−/−	5.2	+/−	30.4
14	M/52	SCPL	+/−	15.8	+/−	24.8
15	M/62	SCPL	+/−	16.0	+/−	35.4

SCPL = supracricoid partial laryngectomy; TL = total laryngectomy; PCNA = proliferating cell nuclear antigen

Proliferating cell nuclear antigen is an excellent marker of proliferation. Our results demonstrate a significant increase in the PCNA index in recurrent laryngeal cancers following radiotherapy, compared with primary, pre-radiotherapy cancers. This finding is consistent with the report that there is a higher degree of cell proliferation in recurrent prostate cancers following radiotherapy.[5] Recurrent tumour

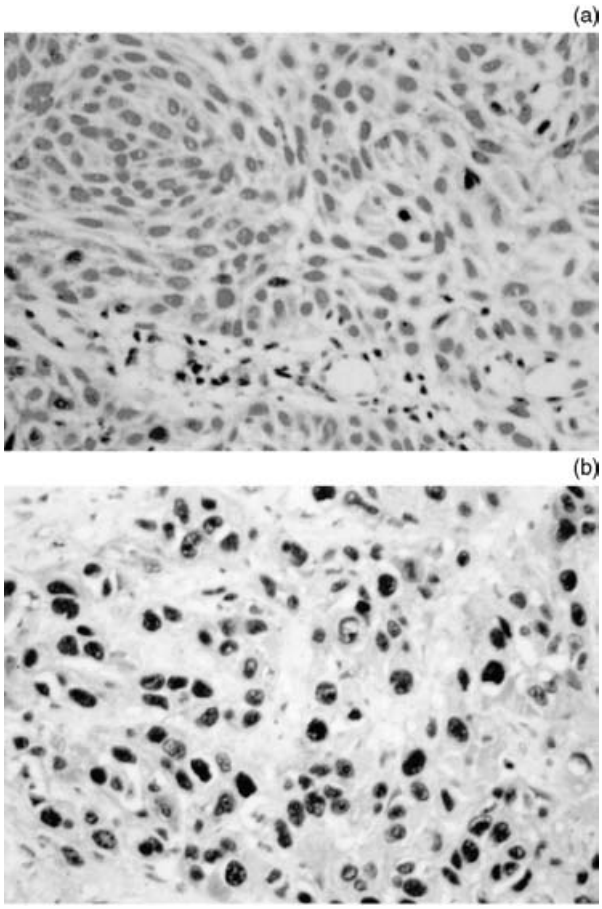

FIG. 1

Immunohistochemical staining of p53. Expression of p53 protein in the recurrent laryngeal cancer tissue after radiation (b) is at a higher intensity than that in the pre-radiation cancer tissue (a) (in case 12, ×400).

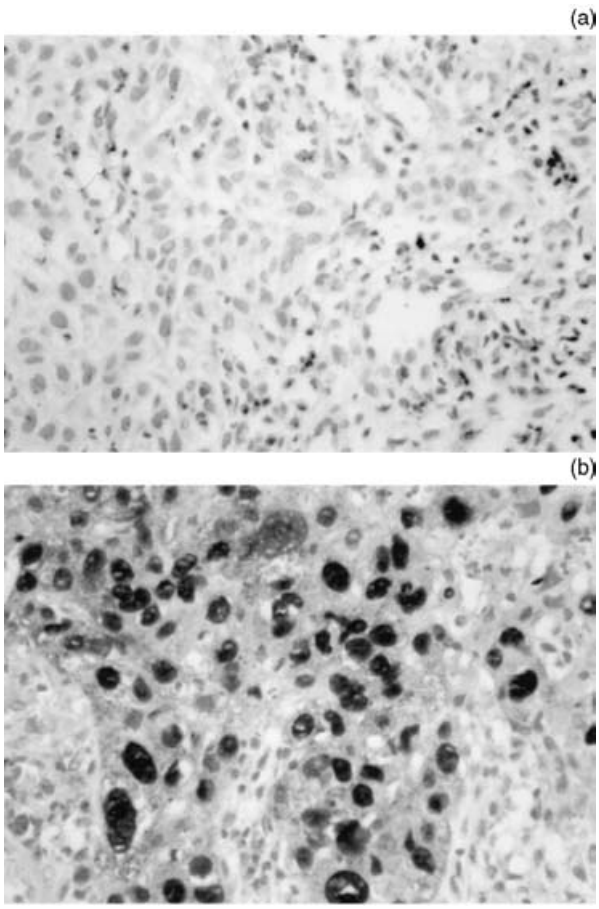

FIG. 2

Immunohistochemical staining of proliferating cell nuclear antigen (PCNA). Expression of PCNA in the recurrent cancer tissue after radiation (b) is at a higher intensity than that in the pre-radiation cancer tissue (a) (in case 13, ×400).

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cells following radiotherapy may receive additional genetic damage, leading to a further loss of growth control and an increase in cellular proliferation rate, compared with tumour cells in the primary, pre-treatment tumour. The increased PCNA expression in recurrent laryngeal cancers after radiation suggests that PCNA expression may be related to sensitivity to radiotherapy. This result is consistent with the finding of Dobros *et al.*[10] that PCNA scores are good indicators of radiation exposure in laryngeal cancer.

Although the bcl-2 gene is involved in the apoptotic pathway, and high bcl-2 expression is fairly well established as a favourable prognostic marker in some carcinomas, the relationship between bcl-2 expression and prognosis in laryngeal cancer has been debated.[11–13] In our study, there was no statistically significant difference in bcl-2 expression between primary, pre-radiotherapy laryngeal cancer and recurrent laryngeal cancer following radiotherapy.

A few studies have examined the biological changes in paired primary and recurrent cancer treated with surgery and/or radiation.[5,14] Although there was no statistically significant alteration of p53 and PCNA in primary and recurrent colorectal adenocarcinoma after surgery with or without radiation, the changes in apoptotic index, p34cdc2 and cyclin D1 were statistically significant.[11] Our results differ from those of Seong *et al.*[14] The possibility that the genetic alteration observed in recurrent cancer after surgery is different from the changes seen after radiation cannot be excluded.

Conclusion

The expression of p53 and PCNA increases in recurrent laryngeal cancer after radiotherapy, compared with that in laryngeal cancer before radiotherapy. Our results suggest that locally recurrent tumours after radiotherapy may possess a more aggressive phenotype than the primary, pre-radiotherapy tumour. Although the number of specimens was limited, the results in this study provide a better understanding of the biological characteristics of recurrent laryngeal cancer after radiation. Future studies with a large number of specimens should help to clarify why recurrent tumours respond poorly to anticancer treatment. This may be the starting point for developing a new therapeutic strategy for the treatment of recurrent cancer after radiation.

- **This study investigated the differences in expression of p53, proliferating cell nuclear antigen (PCNA) and bcl-2 in laryngeal cancer before radiotherapy and in recurrent cancer after radiotherapy, in the same patients**
- **The expression of p53 and PCNA increased in recurrent cancer after radiotherapy. There was no change in expression of bcl-2 after radiotherapy**
- **Recurrent laryngeal cancers became biologically aggressive after radiotherapy**

References

1 Radford IR. The level of induced DNA-double-strand breakage correlates with cell killing after X-irradiation. *Int J Radiat Biol* 1995;**15**:3032–40
2 Laramore GF. Biophysiology and clinical considerations in radiotherapy. In: Cummings CW, Fredrickson JM, Harker LA, Krause CJ, Richardson MA, Schuller DE, editors. *Otolaryngology Head & Neck Surgery*, 3rd ed., St. Louis: Mosby, 1998;88
3 Stamey TA, Ferrari MK, Schmid HP. The value of serial prostate specific antigen determinations 5 years after radiotherapy: steeply increasing values characterize 80% of patients. *J Urol* 1993;**150**:1856–9
4 Wheeler JA, Zagars GK, Ayala AG. Dedifferentiation of locally recurrent prostate cancer after radiation therapy: evidence for tumor progression. *Cancer* 1993;**71**:3783–7
5 Grossfeld GD, Olumi AF, Connolly JA, Chew K, Gibney J, Bhargava V *et al*. Locally recurrent prostate tumors following either radiation therapy or radical prostatectomy have changes in Ki-67 labeling index, p53 and bcl-2 immunoreactivity. *J Urol* 1998;**159**:1437–43
6 De Benedetti VM, Travis LB, Welsh JA, van Leeuwen FE, Stovall M, Clarke EA *et al*. p53 mutations in lung cancer following radiation therapy for Hodgkin's disease. *Cancer Epidemiol Biomarkers Prev* 1996;**5**:93–8
7 Narayana A, Vaughan AT, Gunaratne S, Kathuria S, Walter SA, Reddy SP. Is p53 an independent prognostic factor in patients with laryngeal carcinoma? *Cancer* 1998;**82**:286–91
8 Koch WM, Brennan JA, Zahurak M, Goodman SN, Westra WH, Schwab D *et al*. p53 mutation and locoregional treatment failure in head and neck squamous cell carcinoma. *J Natl Cancer Inst* 1996;**88**:1580–6
9 Lera J, Lara PC, Perez S, Cabrera JL, Santana C. Tumor proliferation, p53 expression, and apoptosis in laryngeal carcinoma: relation to the results of radiotherapy. *Cancer* 1998;**83**:2493–501
10 Dobros W, Rys J, Niezabitowski A, Olszewski E. The prognostic value of proliferating cell nuclear antigen (PCNA) in the advanced cancer of larynx. *Auris Nasus Larynx* 1998;**25**:295–301
11 Silvestrini R, Venerooni D, Daidone MC, Daidone MG, Tomasic G, Squicciarini P *et al*. The Bcl-2 protein: a prognostic indicator strongly to p53 protein in lymph node-negative patients. *J Natl Cancer Inst* 1994;**86**:499–504
12 Friedman M, Lim JW, Manders E, Schaffner AD, Kirshenbaum GL, Tanyeri HM *et al*. Prognostic significance of Bcl-2 and p53 expression in advanced laryngeal squamous cell carcinoma. *Head Neck* 2001;**23**:280–5
13 Lazaris AC, Lendari I, Kavantzas N, Kandiloros D, Adamopoulos G, Davaris P. Correlation of tumor markers p53, bcl-2 and cathepsin-D with clinicopathologic features and disease-free survival in laryngeal squamous cell carcinoma. *Pathol Int* 2000;**50**:717–24
14 Seong JS, Chung EJ, Kim HG, Kim GE, Kim NK, Sohn SK *et al*. Assessment of biomarkers in paired primary and recurrent colorectal adenocarcinoma. *Int J Radiat Oncol Biol Phys* 1999;**45**:1167–73

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