

Expression of XRCC 1 and ERCC 1 proteins in radioresistant and radiosensitive laryngeal cancer

Research Article

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Abbreviations: X-ray repair cross complementing gene, (XRCC 1); Chinese hamster ovary, (CHO); Excision repair complementing defective repair in Chinese hamster, (ERCC 1)

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Summary

Radiotherapy is the principal modality used to treat early stage laryngeal cancer in the UK. Unfortunately treatment failures occur in up to 25% of patients. Subsequent salvage surgery is technically more difficult with the consequences of increased complication and failure rates. The ability to predict radioresistance would significantly improve the poor survival associated with this disease. The efficiency of DNA repair is one of the critical determinants of cell fate following radiotherapy. Using immunohistochemical techniques we examined the expression of DNA repair proteins XRCC 1 and ERCC 1 in 108 pre-treatment laryngeal biopsy samples. All tumours were treated with single modality radiotherapy with curative intent. The group comprised 54 radioresistant and 54 radiosensitive tumours matched for T stage and smoking history. 'Normal' expression of both XRCC 1 and ERCC 1 was significantly associated with radioresistant tumours ($p < 0.001$), with an accuracy of 69% in predicting radiotherapy treatment failure and a low false positive rate of 12%. Patients with predicted radioresistant tumours could be offered conservative 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.

I. Introduction

Radiotherapy used as a single treatment modality can be an effective cure for early stage (T1 and T2) laryngeal tumours. Unfortunately radiotherapy treatment failures do occur: approximately 10% of patients with stage I disease (Klintonberg et al, 1996) and 25% of patients with stage II disease (Fernberg et al, 1989) do not respond to radiotherapy. These observations demonstrate that the TNM system, although widely used as the basis for patient cancer management, cannot always predict an individual tumour's response to radiotherapy.

If a patient fails radiotherapy, a total laryngectomy is the only treatment option that can offer a cure. However, tumour progression may well have occurred adversely affecting patient prognosis still further. The subsequent loss of the larynx will have a significant psychological impact upon the patient and operating in a previously irradiated field results in increased surgical failure and complication rates (McLaughlin et al, 1996). The ability to

predict radiotherapy response at an early stage would improve morbidity and mortality associated with laryngeal cancer.

Due to the essential nature of DNA for genetic inheritance all organisms have evolved mechanisms to recognise and respond to DNA damage. Following radiation-induced DNA damage, cells either undergo cell cycle arrest, to facilitate DNA damage repair, or apoptosis (Shiloh 2003). The efficiency of DNA repair is one of the critical determinants of cell fate following radiotherapy (Polischouk et al, 2001). Base and nucleotide excision repair mechanisms are particularly important in the repair of DNA strand breaks caused by radiotherapy. The DNA repair capacity varies between individuals as a result of inheritance, environmental factors and physiological factors (Scully et al, 2000).

X-ray repair cross complementing gene (XRCC 1) is a key factor involved with DNA strand repair following ionising irradiation. The Chinese hamster ovary (CHO) mutant cell line EM9 has no detectable levels of XRCC 1

and is highly sensitive to ionising irradiation. The molecular basis for this sensitivity was characterised by decreased single stranded DNA break repair (vanAnkeren et al, 1988), reduced recombination repair (Hoy et al, 1987) and increased double stranded DNA breaks (Green et al, 1992). Subsequent expression of XRCC 1 complements the deficiency of the radiosensitive mutant CHO cell line EM9, implicating its involvement with the cells radiation response (Jeggo et al, 1991).

Excision repair complementing defective repair in Chinese hamster (ERCC 1) is essential for nucleotide excision repair in mammalian cells (Westerveld et al, 1984). The CHO mutant cell line 43-3B that has lost ERCC 1 expression is sensitive to UV irradiation. When ERCC 1 was stably transfected into the 43-3B cell line the radiation repair defect in the CHO mutant cells was corrected (Bohr et al, 1988).

Loss of expression of DNA repair proteins that fix the damage caused by ionising radiation may be associated with radiosensitive laryngeal cancer. On the basis of the above observations, the protein expression of XRCC 1 and ERCC 1 was investigated in radioresistant and radiosensitive cohorts of laryngeal cancer patients. It is hypothesised that tumour cells with reduced expression of XRCC 1 or ERCC 1 are radiosensitive at the beginning of radiotherapy treatment and that subsequent fractionated radiotherapy selects out radioresistant clones resulting in the observed clinical tumour recurrence.

II. Materials and methods

A. Samples

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 55Gy in 20 fractions or 60Gy 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 and smoking history. The groups were very similar, with no significant difference with regards to laryngeal sub site, tumour differentiation and gender. Tumours were staged according to the TNM classification (Greene & Sobin 2002) and all were clinically N0 and M0 at the time of treatment.

The radioresistant group consisted of 54 patients: 37 stage T1 and 17 stage T2 laryngeal squamous cell carcinomas (**Table 1**). The criteria for a radioresistant tumour were:

- 1) The radiotherapy had to be given as a single modality treatment with curative intent for a biopsy-proven squamous cell carcinoma of the larynx and
- 2) 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 54 patients: 37 stage T1 and 17 stage T2 squamous cell carcinomas of the larynx. The criteria for a radiosensitive tumour were:

- 1) The radiotherapy had to be given as a single modality treatment with curative intent for a biopsy proven squamous cell carcinoma of the larynx and
- 2) Post treatment, patients had a minimum follow up of 3 years with no evidence of a recurrent laryngeal tumour.

Tissue sections (4µm) were cut from pre-treatment archival tissue blocks of all tumours. Immunohistochemistry as previously described was used to detect XRCC 1 and ERCC 1 on the tissue sections (Cawkwell et al, 1999). Both monoclonal antibodies localised to the nuclear compartment of the cell. In brief, antigen retrieval was performed using pressurised heat retrieval. XRCC 1 was detected using a mouse monoclonal antibody (100µl) anti XRCC 1 (Neomarkers, Fremont, USA, clone 33-2-5) at a dilution of 1:40 with 0.2x casein and ERCC 1 was detected using a mouse monoclonal antibody (Neomarkers clone 8F1) at a dilution of 1:100. The antibodies were added to each tissue section and incubated at room temperature for two hours. A negative control was included using 100µl of 0.2x 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.

B. Marker assessment

No recognised scoring systems for XRCC 1 or ERCC 1, detected by immunohistochemistry, have been published. A proposed marking scheme based on the staining pattern of XRCC 1 and ERCC 1 in 'normal' squamous epithelium, from a test series of stage T3/T4 laryngeal tumours, has been used here. 'Normal' squamous epithelium uniformly stained for both markers. Reduced expression of a marker was deemed to occur if 50% or less of the tumour stained. The 50% cut off was decided upon after assessing the level of ERCC 1 marker expression by one observer in 108 tumour sections using cut off points of, 50%, 25% and 5% and 1%, for a negative result (**Table 2**). A 50% cut off for a reduced marker expression was chosen due to its significant discrimination between the radioresistant and radiosensitive tumours as well achieving a high level of concordance between observers. A similar subjective 50% cut off scoring system for reduced expression of proteins involved in DNA repair has also been reported for laryngeal cancer (Condon et al, 2002). Intensity of tumour staining was not used as a basis for scoring due to potential variations in clinical specimen fixation that affect intensity (Fisher et al, 1994).

Two independent assessors blinded to the final outcome scored XRCC 1 and ERCC 1 throughout the whole biopsy section. If 50% or less of the tumour stained throughout the whole tissue section, reduced expression was recorded. As the whole biopsy section was assessed, in order to reduce a further sampling error of the whole tumour, the scoring was subjective. The two independent assessors had complete agreement in 73% of the XRCC 1 cases and reached consensus agreement after consultation in the remaining cases. For ERCC 1 the two assessors had complete agreement in 77% of the cases and consensus agreement in the remaining cases. In an attempt to validate the consensus results one of the assessors re-scored the markers, once again in a blinded manner and achieved a 94% accuracy when compared with the consensus result. This suggests that a reproducible marking system has been used.

C. Statistics

Chi-Squared and McNemar 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 less than 0.05 were considered significant. Marker accuracy, sensitivity and specificity were calculated as previously described (Greenhalgh, 1997).

III. Results

Representative immunohistochemical staining of

Table 1:
Laryngeal cancer patient characteristics

	Radioresistant (n=54)	Radiosensitive (n=54)
Mean Age, years (SD)	64 (9.5)	64 (9.8)
Patient gender:		
-Male	46	42
-Female	8	12
Mean time to recurrence (months)	6 (2-12)	-
T Stage:		
-T1	37	37
-T2	17	17
Laryngeal sub site:		
-Glottic tumours	50	48
-Supraglottic tumours	4	6
Tumour differentiation:		
-Well	16	17
-Moderate	32	27
-Poor	6	10

Table 2:
ERCC1 expression in 108 laryngeal cancers using different positive cut off points

% of positively Stained tumour cells	Radioresistant (n=54)	Radiosensitive (n=54)	p value*
50%	17%	41%	0.005
<25%	11%	33%	0.02
<5%	7%	11%	0.9
<1%	0%	4%	0.7

*Chi Squared 95% two-sided significance

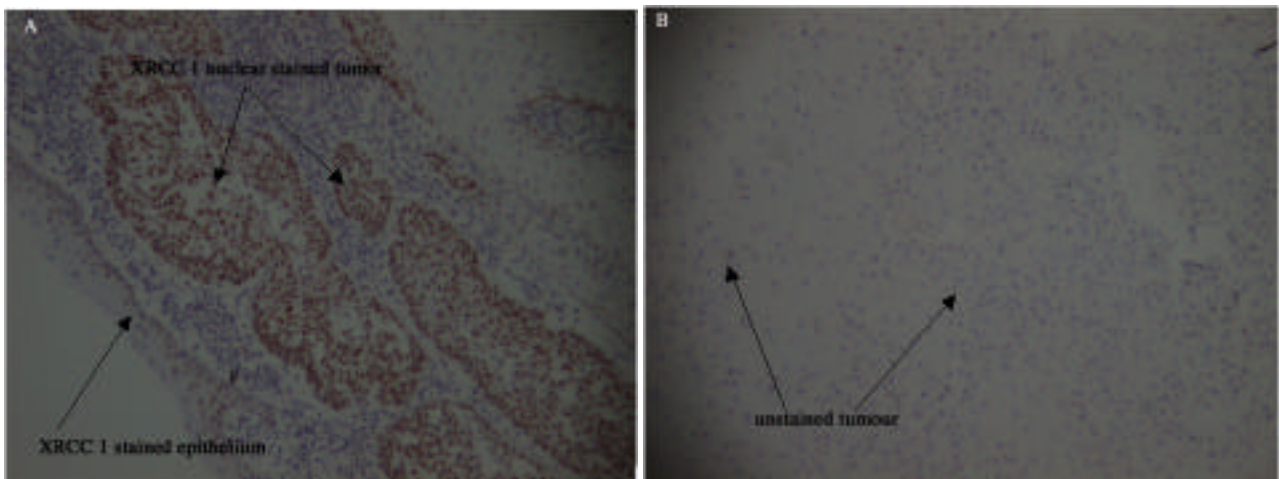


Figure 1: Immunolocalisation of XRCC 1 on a laryngeal biopsy tissue section. **A** – Radioresistant sample demonstrating >50% of tumour cells staining for XRCC 1. Nuclear staining of squamous cell carcinoma radioresistant tumour cells with XRCC 1. Staining of the normal squamous epithelium acts as an internal positive control. **B**. Radiosensitive tumour demonstrating < 50% of the tumour nuclei have stained with XRCC 1. Magnification x100

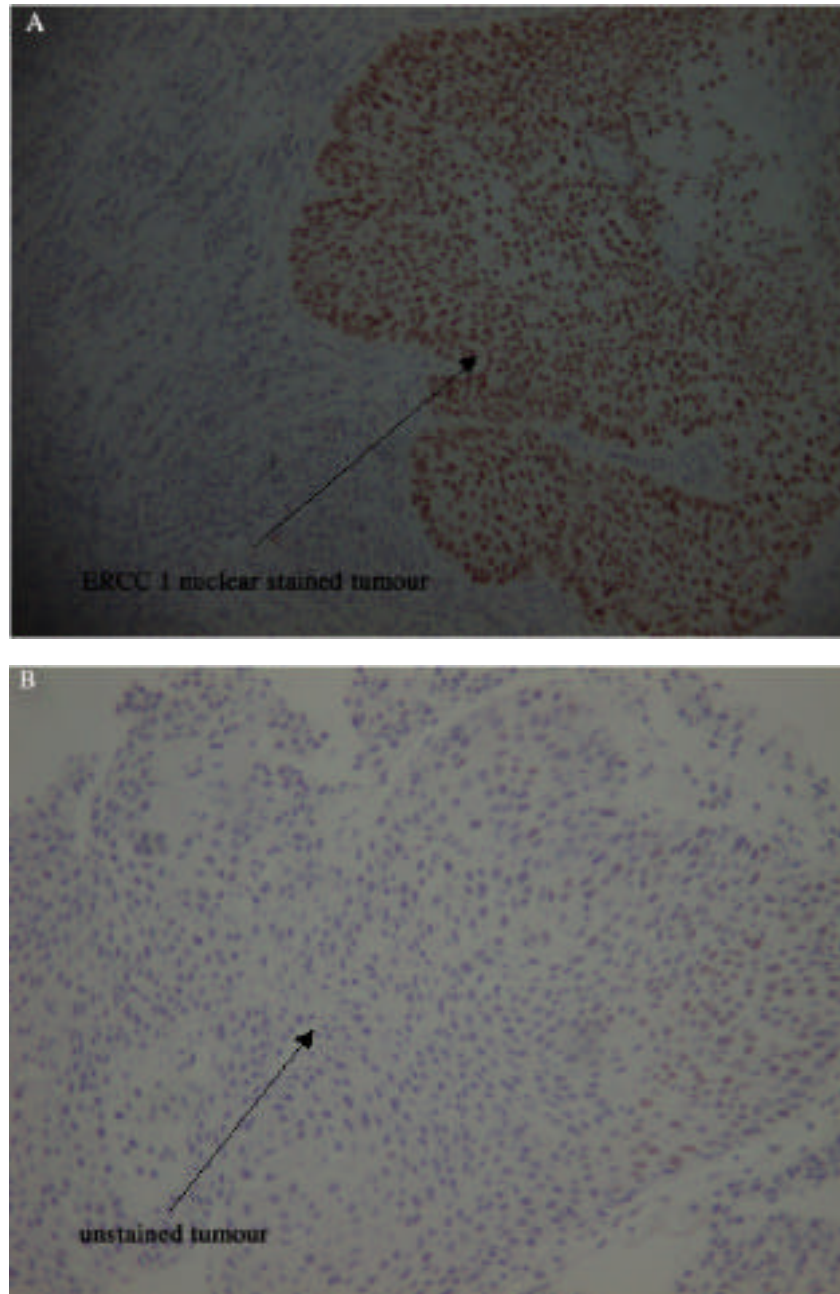


Figure 2: Immunolocalisation of ERCC 1 on a laryngeal biopsy tissue section **A.** Radioresistant tumour biopsy demonstrating nuclear staining of squamous cell carcinoma cells with ERCC 1. The majority of tumour nuclei have stained. **B.** Radiosensitive tumour demonstrating that < 50% of the tumour nuclei have stained with ERCC 1. Magnification x100

ERCC 1 and XRCC 1 proteins is shown in **Figures 1 and 2.**

XRCC 1 and ERCC 1 were both localised to the nucleus of tumour and the normal squamous epithelial cells. The staining of the 'normal' squamous epithelium served as an internal positive control implying that the tissue antigens under investigation had been preserved in a detectable form during the fixation process. Reduced XRCC 1 expression was observed in 37% of radioresistant compared with 57% of radiosensitive tumours (**Table 3**). Reduced ERCC 1 expression was exhibited in 18% of radioresistant compared with 46% of radiosensitive tumours. These results were significant, $p=0.034$ and $p=0.002$ respectively.

In the radioresistant cohort ($n=54$) 61% of the tumours had normal expression of both XRCC 1 and ERCC 1 ($p=0.006$) compared with only 24% of the tumours in the radiosensitive cohort (**Table 4**).

If expression, in >50% of tumour cells, of both XRCC 1 and ERCC 1 is used as a predictive marker for radiotherapy outcome in early stage laryngeal cancer, it has an accuracy of 69% and a low false positive rate of 12% (**Table 5**).

IV. Discussion

At present there are no studies evaluating DNA repair protein expression in radioresistant head and neck cancer. We report that loss of XRCC 1 and ERCC 1 expression correlates with radiotherapy outcome in laryngeal cancer. In order to reduce confounding variables we have limited the study to the larynx, the largest head and neck region affected by cancer in the UK and applied a strict definition of radioresistance. By stipulating that recurrences had to occur at the original anatomical site following radiotherapy occult metastasis that occur in regional lymph nodes will not be erroneously counted as a recurrence. Also the recurrence had to be of a similar histology and occur within 12 months of finishing the course of radiotherapy. This will exclude the majority of second primary tumours, that are common in the head and neck region (Holland et al, 2002). If these second primary tumours were not excluded they would be erroneously interpreted as a radiotherapy recurrence.

By close matching of the tumour groups, variables such as TNM stage and smoking history were removed as possible confounding variables in the reported results. The analysis was limited to early stage laryngeal tumours (T1 or T2 N0 and M0) that are widely recognised as tumours that can be treated with single modality radiotherapy or

partial laryngeal surgery with equal effect. Our results demonstrate that 57% of radiosensitive tumours had reduced expression of XRCC 1 compared with 37% in the radioresistant group. For ERCC 1 46% had reduced expression compared with only 18% in the radioresistant group. These results suggest that reduced tumour DNA repair capacity is associated with radiosensitivity in early stage laryngeal cancer, an observation that has been reported in the N10 radioresistant cell line (Yanagisawa et al, 1998). The human DNA repair gene XRCC 1 was over expressed in a human radiosensitive cell line, KB. Compared with its radiosensitive counterpart, as determined by Northern blot analysis, constitutively N10 KB cells showed higher expression of XRCC 1 mRNA than did the parental KB cells. After irradiation of both cell lines with 4 Gy the N10KB cell line showed enhanced survival and increased XRCC 1 mRNA compared with the KB cell line.

Labudova et al (1997) characterised the expression of XRCC 1 mRNA in two genetically well-defined animal systems differing in their known sensitivity to ionising radiation. The radioresistant C3H He/Him mice had higher levels of XRCC 1 mRNA than the radiosensitive BALB/c/J Him mice before any radiation.

Table 3

XRCC1 and ERCC 1 expression in 54 radioresistant and 54 radiosensitive T1 and T2 laryngeal cancers

	Radioresistant (n=54)	Radiosensitive (n=54)	p value*
XRCC 1 expression			
50%	20 (37%)	31 (57%)	0.034
>50%	34 (63%)	23 (43%)	
ERCC 1 expression			
50%	10 (18%)	25 (46%)	0.002
>50%	44 (82%)	29 (54%)	

*Chi Squared 95% two-sided significance

Table 4

Co-expression of XRCC 1 and ERCC 1 in 54 Radioresistant and 54 Radiosensitive T1 and T2 laryngeal cancers

Radioresistant tumours (n=54)	XRCC 1 expression		p value*
	50%	>50%	
ERCC 1 expression			
50%	9	1	0.006
>50%	11	33	
Radiosensitive tumours (n=54)	XRCC 1 expression		p value*
	50%	>50%	
ERCC 1 expression			
50%	15	10	0.327
>50%	16	13	

*McNemar test, two sided significance

Table 5

Predictive value of both XRCC 1 and ERCC 1 expression as a marker of radiotherapy outcome in 108 patients with early stage laryngeal cancers

XRCC 1 and ERCC 1 >50% expression	Final outcome of therapy	
	Tumour recurrence =54	Tumour free =54
Positive =46	True +ve =33	False +ve =13
Negative =64	False -ve =21	True -ve =41
Sensitivity	61%	
Specificity	76%	
Positive predictive value	61%	
Negative predictive value	66%	
Accuracy	69%	
False positive	12%	
True positive	31%	

Following 4Gy the radioresistant mice significantly increased the levels of XRCC 1 mRNA compared with the radiosensitive mice. In summary XRCC 1 appears to be associated with cellular radioresistant in both animal and human systems. XRCC 1 and ERCC 1 have been chosen as possible discriminators of radiation sensitivity based upon the observations stated above and in the introduction. The fact that both DNA repair proteins had a significantly reduced expression in the radiosensitive tumours may suggest that there is a global decrease in DNA repair. Intuitively this would be expected with tumours that are sensitive to radiation damage. It may be that there is an upstream regulator of DNA strand breaks following radiation damage that better correlates with radiation response.

The association of both XRCC1 and ERCC 1 expression in the nuclei of at least 50% of tumour cells in the pre-treatment biopsy material may be used as a prognostic marker predicting radiotherapy treatment failure with an accuracy of 69%. The 31% of patients with radioresistant T1 or T2 laryngeal cancer and are XRCC 1 and ERCC 1 positive could be offered conservative 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 (Wilson 2002). 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 receive unnecessary radiotherapy. Equally there will be no detrimental effect to the 12% of patients with a false positive result who would be offered partial laryngeal surgery instead of radiotherapy.

Predicting radiotherapy treatment failure using pre-treatment biopsy material would be a significant clinical advance in the treatment of laryngeal cancer. At present radioresistant laryngeal T1 or T2 tumours cannot be predicted. Using XRCC 1 in combination with ERCC 1 can predict 31% of the radioresistant cases.

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