

The lack of correlation between proliferation (Ki-67, PCNA, LI, Tpot), p53 expression and radiosensitivity for head and neck cancers

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Summary A study was made of the relationship between measurements of radiosensitivity versus proliferation and p53 status in head and neck cancers. Inherent tumour radiosensitivity was assessed as surviving fraction at 2 Gy (SF2) using a clonogenic soft agar assay ($n = 77$). The results were compared to data on proliferation obtained by both flow cytometry (labelling index (LI), the potential doubling time (Tpot) $n = 55$) and immunohistochemistry (Ki-67 and PCNA; $n = 68$), together with immunohistochemical p53 expression ($n = 68$). There were no overall significant differences in the median values of the various parameters analysed for the different sites within the head and neck region, disease stages, grades of tumour differentiation or nodal states. A subgroup analysis showed that oropharyngeal ($n = 22$) versus oral cavity ($n = 35$) tumours were more radiosensitive ($P = 0.056$) and had a higher Ki-67 index ($P = 0.001$). Node-positive tumours had higher LI ($P = 0.021$) and a trend towards lower Tpot ($P = 0.067$) values than node-negative ones. No correlations were seen between SF2 and any of the parameters studied. The long-standing dogma of an increased radiosensitivity of rapidly proliferating cells in contrast to slowly proliferating cells was not confirmed. The study shows that parallel measurements of different biological markers can be obtained for a large number of patients with head and neck cancers. The independence of the various parameters studied suggests that there may be potential for their combined use as prognostic factors for the outcome of radiotherapy.

Keywords: proliferation; p53; radiosensitivity; head and neck cancer

Head and neck cancer is a disease which is usually treated with radiotherapy, surgery alone or a combination of surgery and pre- or post-operative radiotherapy. Treatment decisions are usually based not only on results from previous trials, patient parameters (performance status, age, weight loss, other co-existing diseases) and clinicopathological factors (site, TNM status, grade of differentiation) but also on local treatment traditions. The latter will favour either radical radiotherapy, surgery, or a combined modality approach. The advantages of radiotherapy over surgery in the management of the disease is in the possibility of organ preservation and maintenance of important functions (e.g. speaking, swallowing). The advantages of surgery are short overall treatment times and less radiotherapy-specific morbidity (e.g. dry mouth, taste alteration and radionecrosis). The potential value of finding biological predictive factors is in being able to offer an individualized approach to treatment to select the most appropriate type of therapy. In theory this should lead to not only increases in local control but also reductions in treatment-specific morbidity.

Regarding biological predictive factors for radiotherapy response, tumour proliferation is probably the most widely studied parameter, in particular the potential doubling time (Tpot) of a

tumour (Bourhis et al, 1993; Begg et al, 1995; Zackrisson et al, 1997). However, a recent meta-analysis of 210 aneuploid tumours treated with conventional radiotherapy showed that labelling index (LI) but not Tpot was a significant predictor of local control (Begg, 1997). Nevertheless, there are statistical and methodological questions regarding the appropriateness of pooling multi-centre data and using Tpot as a continuous variable (Coucke et al, 1998). There is, therefore, still interest in measuring proliferation in head and neck cancers.

Another potential biological prognostic parameter for radiotherapy response is tumour radiosensitivity, usually measured as surviving fraction at 2 Gy (SF2) (West, 1995). There is good evidence that in-vitro clonogenic assays reflect the radiosensitivity of tumour in vivo (reviewed in West, 1995). In particular, in cervix tumours SF2 has been shown to be an independent prognostic factor for radiotherapy response (West et al, 1997). The results from studies on head and neck cancer, however, have been disappointing. SF2 measured using the CAM (cell adhesive matrix) assay on tumour specimens from patients treated by radiotherapy and surgery showed a trend for patients with in vitro radio-resistant versus -sensitive tumours to have a worse outcome but the difference was not significant (Brock et al, 1990). Using the same assay, a study on patients treated predominantly by radiotherapy alone showed that tumour radiosensitivity was significantly associated with treatment outcome (Girinsky et al, 1994) but in a recent update of the data the significance was lost (Eschwege et al, 1997). Thus, it is yet to be established whether

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radiosensitivity measurements obtained using a clonogenic assay are prognostic for radiotherapy outcome in head and neck cancers. Although we have carried out a large study measuring tumour radiosensitivity using a soft agar assay (Björk-Eriksson et al, 1998), analysis with respect to treatment outcome awaits adequate patient follow-up.

The *TP53* gene is important in the pathogenesis of cancer and mutations in it are the most common found in human cancers. The *TP53* gene also has a clear role to play in regulating cell proliferation and radiosensitivity and as a checkpoint protein transactivating other genes (Yin et al, 1992; Bristow et al, 1996). With the possible exceptions of laryngeal- and oropharyngeal carcinomas (Sauter et al, 1992; Bradford et al, 1995; Narayana et al, 1998), most studies using immunohistochemistry have not shown any correlation between p53 overexpression and local control or survival in head and neck cancers (Field et al, 1993; Raybaud-Diogene et al, 1997). Therefore, it is of interest to study the relationship between p53 overexpression versus inherent tumour radiosensitivity and proliferation.

There is little information on the relationship between radiosensitivity and proliferation in the same patient, and between SF2 and p53 expression in primary tumours. Therefore, the aims of this study were to examine the relationship between SF2, various proliferation parameters (Ki-67, PCNA, LI, Tpot) and p53 expression for a large number of patients with head and neck cancers.

MATERIALS AND METHODS

Specimens

All tumour material was obtained following informed consent. Biopsy or surgical specimens were taken from patients with previously histopathologically confirmed and untreated primary squamous cell- or undifferentiated carcinoma of the head and neck region. Samples were collected for radiosensitivity testing, flow-cytometric analysis and routine histopathology, including later immunohistochemical staining. The specimens were divided and portions were placed in transport medium (Björk-Eriksson et al, 1998), 70% ethanol and 4% formaldehyde for the various analyses to be carried out later. All the material was from primary lesions and no selection criteria were used regarding tumour site of origin within the head and neck region or TNM-stage (Spiessl et al, 1990). The specimens were collected from the Department of Otorhinolaryngology Head and Neck Surgery, Sahlgrenska University Hospital in Gothenburg, Sweden.

Radiosensitivity

Tumour radiosensitivity was measured on primary biopsies as the surviving fraction following an acute exposure of 2 Gy in vitro (SF2) using a soft agar clonogenic assay. The materials and methods used have been described in detail elsewhere together with a description of the immunohistochemical characterization of the malignant epithelial origin of colonies (Björk-Eriksson et al, 1998).

Immunohistochemistry

Formalin-fixed and paraffin-embedded sections, 5- μ m thick, were stained using the DAKO Techmate500-1000™ automated system. The following antigens (all DAKO™) and dilutions were used:

Table 1 Description of clinical parameters

Parameter	Group	n
Sex	Male	59
	Female	18
Site	Oral	35
	Oropharynx	22
	Nasopharynx	8
	Hypopharynx	3
	Larynx	7
Stage	Sinonasal	2
	II	8
	III	14
Nodes	IV	54
	Negative (N ⁺ = 0)	44
	Positive (N = 1-3)	32
Histology	SCC	77
	Poor	22
	Moderate	38
	Well	7
	Undifferentiated	3

^aRefers to TNM classification according to UICC. SCC, squamous cell carcinoma.

Table 2 Summary of data

Variable	n	Mean \pm 1 s.d.	Median	Range
Age (years)	77	63 \pm 14	63	19-94
SF2	77	0.46 \pm 0.21	0.40	0.10-1.00
p53 (%)	68	18 \pm 27	2	0-85
Ki-67 (%)	67	21 \pm 14	18	2-60
PCNA (%)	68	21 \pm 23	14	0-92
LI (%)	55	8 \pm 5	7	1-24
Tpot (h)	50	128 \pm 117	107	11-660

1/500 anti-human p53 mouse monoclonal antibody (clone DO-7), 1/100 anti-Ki-67 rabbit polyclonal antibody (reactivity similar to MIB-1), and 1/50 anti-PCNA mouse monoclonal antibody (clone PC-10). A microwave antigen retrieval step was used for p53 and Ki-67 only. Both primary and secondary antibodies were incubated for 25 min at room temperature. For Ki-67 and PCNA, normal human pharyngeal tonsil was used as a positive control, and for p53, a known positive human rectal carcinoma served as the positive control. The presence of carcinoma and the number of immunohistochemically stained cells were scored under a light microscope by two independent observers using a 100 squared grid and a magnification of \times 400. All viable nucleated tumour cells at the 121 crossings of the grid were counted and scored as positive or negative. When there was sufficient tumour material, this procedure was repeated five times for each observer to give a total of up to 1210 cells scored. The percentage of positive nuclei was used as a measure of antigen expression.

Flow cytometry

Administration of bromodeoxyuridine (BrdU) was carried out following approval from the Ethical Committee of the University of Gothenburg, Sweden. A short time intravenous (i.v.) infusion of 250 mg BrdU dissolved in 100 ml natural saline was given approximately 6 h before biopsy. Nuclei extraction, DNA staining, analysis on the FACS scan flow cytometer (Becton-Dickson) and

Table 3 Distribution within the different tumour sites of the head and neck

Site	SF2	p53 (%)	Ki-67 (%)	PCNA (%)	LI (%)	Tpot (h)
Oral cavity n =	0.51 ± 0.23 35	19 ± 27 31	15 ± 12 31	21 ± 24 31	7 ± 5 24	121 ± 82 20
Oropharynx n =	0.38 ± 0.19 22	16 ± 27 21	27 ± 14 21	25 ± 21 21	9 ± 5 14	124 ± 116 13
Nasopharynx n =	0.45 ± 0.18 8	17 ± 25 5	21 ± 14 5	5 ± 7 5	7 ± 4 7	173 ± 218 7
Hypopharynx n =	0.28 ± 0.06 3	18 ± 30 3	35 ± 10 3	37 ± 41 3	16 ± 7 3	32 ± 24 3
Larynx n =	0.45 ± 0.12 7	8 ± 13 6	23 ± 11 5	16 ± 12 6	8 ± 5 5	154 ± 105 5
Sinonasal n =	0.68 ± 0.37 2	77 ± 1 2	26 ± 34 2	4 ± 3 2	6 ± 2 2	144 ± 60 2

All values represent mean ± one standard deviation.

Table 4 Subset analysis according to stage (II, III and IV) and tumour differentiation (Diff) (Poor, Mod and Well)

Stage/ Diff.	SF2	p53 (%)	Ki-67 (%)	PCNA (%)	LI (%)	Tpot (h)
II n =	0.59 ± 0.23 8	12 ± 18 7	13 ± 8 7	12 ± 13 7	5 ± 4 3	120 ± 13 2
III n =	0.40 ± 0.15 14	27 ± 33 13	23 ± 16 13	13 ± 19 13	8 ± 4 10	109 ± 76 7
IV n =	0.44 ± 0.20 54	17 ± 27 47	22 ± 14 46	24 ± 24 47	8 ± 5 41	133 ± 127 40
Poor n =	0.46 ± 0.20 22	15 ± 26 20	25 ± 17 20	21 ± 20 20	95 16	121 ± 114 15
Mod n =	0.44 ± 0.22 38	26 ± 31 34	21 ± 14 34	21 ± 21 34	8 ± 5 25	119 ± 83 21
Well n =	0.44 ± 0.18 7	5 ± 8 7	17 ± 11 7	33 ± 38 7	12 ± 9 4	73 ± 52 4

All values represent mean ± one standard deviation. Stage II, III and IV refers to the TNM classification according to UICC (Spiessl et al, 1990). Tumour differentiation: Poor = poorly differentiated squamous cell carcinoma; Mod = moderately differentiated squamous cell carcinoma; Well = well-differentiated squamous cell carcinoma.

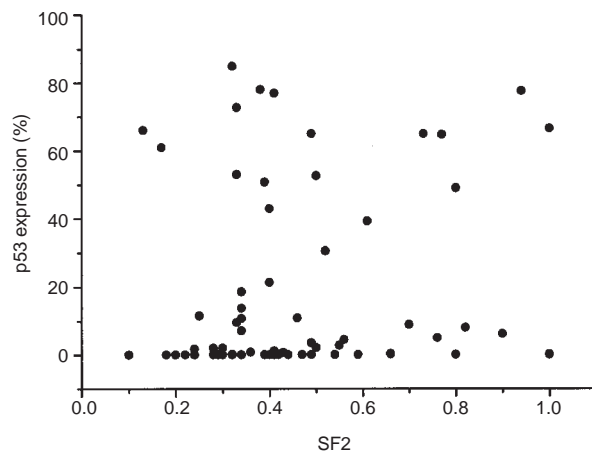
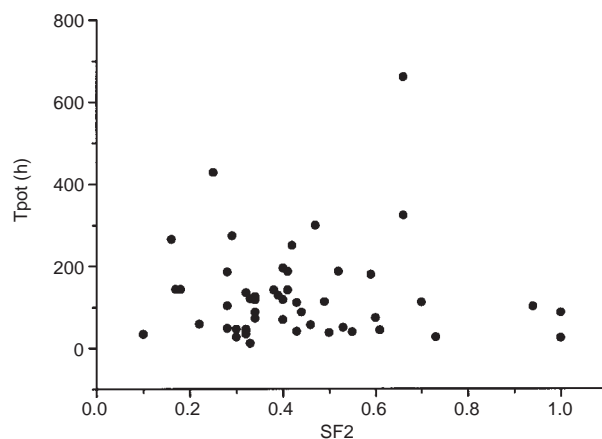
the calculations of LI as well as Tpot were performed as described previously (Lydén et al, 1995).

Statistical analysis

Correlations between variables were determined using Spearman's rank correlation. Subset analyses were carried out with groups of more than 30 patients, which had 80% power to detect a significant correlation. The Mann-Whitney *U*-test and Kruskal-Wallis one-way analysis of variance were used to test for the level of significance between independent variables. A significance level of 0.05 was used throughout.

RESULTS

SF2 values were obtained for 77 patients with head and neck malignancies. Table 1 summarizes the distribution of the patients according to sex, site, stage, nodal status and histology. Staging information was not available for one patient and seven tumours were classified as squamous cell carcinoma only with no further

**Figure 1** The lack of relationship between tumour radiosensitivity (SF2) and the percentage of cells stained p53 positive for 68 head and neck tumours ($r = 0.17$, $P = 0.17$)**Figure 2** The lack of relationship between tumour radiosensitivity (SF2) and proliferation (Tpot) for 50 cancers of the head and neck ($r = 0.07$, $P = 0.64$)

indication of the degree of differentiation. Table 2 summarizes the data for tumour radiosensitivity (SF2), patient age and the various immunohistochemical (p53, Ki-67, PCNA) and flow cytometry (LI, Tpot) parameters. Immunohistochemical scoring of the three antibodies studied were well-correlated for the two independent observers: p53 ($r = 0.90$, $P < 0.001$), Ki-67 ($r = 0.81$, $P < 0.001$) and PCNA ($r = 0.88$, $P < 0.001$). In contrast, there was considerable inter-tumour variability in immunohistochemical expression with coefficients of variation of 150%, 67% and 151% for p53, Ki-67 and PCNA respectively.

Before correlations between variables were obtained, an analysis was made of the distribution of values within the different sites of the head and neck region, disease stages, grades of tumour differentiation and tumour nodal status. Using the Kruskal-Wallis non-parametric test, there were no significant differences in the median values of the various parameters for the different sites within the head and neck region (Table 3). A subgroup analysis was made of the two most frequently-presenting sites. Oropharyngeal tumours were more radiosensitive ($P = 0.056$) and had a higher Ki-67 index ($P < 0.001$) than carcinomas of the oral cavity. There were no significant differences in the median values of the various parameters for the different disease stages (Table 4; $P > 0.14$) and grades of

tumour differentiation (Table 5; $P > 0.44$). Node-positive tumours had higher values for LI ($P = 0.021$) and tended to have lower values for Tpot ($P = 0.067$) than node-negative cancers.

The relationships between variables were investigated using Spearman's rank correlation. No significant correlations were seen between SF2 and Ki-67 index ($r = -0.19$, $P = 0.13$), PCNA index ($r = -0.10$, $P = 0.40$), or LI ($r = -0.10$, $P = 0.46$). The Figures illustrate the lack of relationship between SF2 versus p53 expression (Figure 1) and Tpot (Figure 2). The analyses were repeated for the largest clinical subgroups. No significant correlations were also seen when the data were reanalysed for oral cavity ($P > 0.061$), stage IV disease ($P > 0.49$) or moderately differentiated tumours ($P > 0.28$) only.

Significant correlations were seen between some of the other parameters. As expected, LI and Tpot were highly significantly correlated ($n = 50$, $P < 0.001$). A significant correlation was also seen between the expression of the two proliferative markers, Ki-67 and PCNA ($n = 67$, $P = 0.036$). There were no significant relationships between the level of p53 expression and any of the proliferation parameters.

DISCUSSION

The individualization of radiotherapy using biological predictive tests alongside the traditional patient and clinicopathological parameters could lead to not only increased local control, but also less morbidity. The prognostic factors of most interest are those which are known to affect radiotherapy outcome, namely tumour proliferation, radiosensitivity and oxygenation, and the intrinsic radiosensitivity of normal cells. There have now been numerous studies that have examined the potential of all these parameters as prognostic factors for radiotherapy outcome (Begg, 1997). It may be, however, that the greater discrimination of radiotherapy outcome groups will be via multiple biological tests. For example, large differences in survival and local control probabilities have been shown by combining two biological parameters (Levine et al, 1995; Raybaud-Diogene et al, 1997; West et al, 1998). It is of interest, therefore, to examine the relationship between potential prognostic factors in order to show both their independence and the feasibility of carrying out multiple measurements. It is surprising then that there are little data where measurements have been made of SF2 and Tpot on primary tumours from the same individual. A paper, where Tpot and SF2 had been obtained on only nine patients, highlighted some of the problems in carrying out parallel measurements such as inadequate sample size and patient refusal (Eschwege et al, 1997).

In the work described here, tumour Tpot measurements were made on 50 out of 77 (65%) patients for whom SF2 data were collected. Reasons for failure to obtain Tpot data were predominantly related to patient refusal and age. Nevertheless, we have clearly demonstrated that it is feasible to make multiple biological measurements. In addition, our work has shown that there is no relationship between the intrinsic radiosensitivity of a tumour and its rate of proliferation, whether measured by flow cytometry or the simpler immunohistochemical methods. This finding is supported by work carried out on cells in culture which has shown that there is no relationship between intrinsic radiosensitivity and the in vitro proliferation rate measured either as cell doubling time (Pekkola-Heino et al, 1994) or as LI and Tpot (Warenius et al, 1994). The lack of relationship between SF2 and Tpot/LI is encouraging for the future parallel assessment of radiosensitivity

and proliferation. It has been suggested that it may be necessary to correct for differences in tumour radiosensitivity before tests based on other radiobiological parameters may have clinical significance (Tucker and Thames, 1989). It will, therefore, be of interest in the future to examine the prognostic significance of LI (and the other proliferation measures) after allowing for differences in tumour radiosensitivity.

Over the past decade there has been considerable interest in measuring the tumour suppressor and regulatory gene p53 product. In cancers of the head and neck, overexpression of p53 has been studied in relation to local control (Wilson et al, 1995; Narayana et al, 1998), survival (Sauter et al, 1992; Field et al, 1993; Nylander et al, 1995) and lately organ preservation (Bradford et al, 1995). In general, p53 expression using immunohistochemistry is not a significant prognostic factor for radiotherapy outcome and the use of functional assay has been suggested (Bristow et al, 1996). However, there is still interest in studying the relationship between tumour p53 expression and radiosensitivity as the findings have been equivocal. In a series of 24 head and neck cancer cell lines, Brachman et al (1993) found no relationship between p53 mutations and SF2. However, in 16 oral cavity carcinoma cell lines, the 11 lines that had a mutated p53 gene were significantly more radiosensitive than those with wild-type p53 (Pekkola-Heino et al, 1996). Studies on other tumour types have yielded different results with some showing a significant association (Siles et al, 1996) and others not (Zaffaroni et al, 1995). In our work we have shown no relationship between SF2 and p53 expression, measured using immunohistochemistry in mixed cancers of the head and neck or in a subsite analysis of 31 oral cavity tumours ($r = 0.24$, $P = 0.20$). One of the principal functions of p53 is the induction of cell cycle arrest. As loss of wild-type expression is associated with cellular growth, a relationship might be expected between p53 expression and proliferation. This was not found either in our study or the work of others on head and neck cancer (Wilson et al, 1995). There are several possible reasons. First, it may be a methodological problem relating to the use of immunohistochemistry rather than mutational analysis. Immunohistochemistry using the DO7 antibody has been reported to have concordance with mutational analysis in 71% of head and neck cancers, while 14% were DO7-positive with no detected mutations and 15% had *TP53* mutations not detected by DO7 (Calzolari et al, 1997). Second, a lack of correlation between p53 expression and proliferation may relate to the variety of different effects mediated via p53. Third, it may be important to investigate a single tumour site within the head and neck. In support of the latter, Bourhis et al (1994) showed a significant correlation between p53 overexpression and short Tpot in 49 oropharyngeal cancers. Regarding the relationship between p53 and proliferation, more information would be gained by carrying out mutational analysis of *TP53* and by concentrating on a single tumour type. Although this is a limitation of the present study, our primary objective was to obtain radiosensitivity measurements on a large number of tumours, and correlate the radiosensitivity data with other biological parameters.

In summary, this work has highlighted the feasibility of carrying out multiple measurements of radiobiological parameters on patients with cancers of the head and neck. No correlations were seen between SF2 and measures of proliferation and p53 expression. All the patients included in this study were treated with radiotherapy, but follow-up times were too short to allow correlations with clinical outcome. However, in the future we will

examine the relationships between the various factors studied and radiation response in vivo.

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