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Proliferating Cell Nuclear Antigen (PCNA) Expression and Nuclear DNA Content in Predicting Recurrence After Radiotherapy of Early Glottic Cancer

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Proliferating cell nuclear antigen (PCNA) is a DNA replication protein maximally elevated in late G1 and S phases of the cell cycle. By using monoclonal antibodies, the expression of PCNA can be quantified and the rate of tumour cell proliferation estimated. The degree of DNA aberration in a tumour cell population reflects its genetic instability and has been implicated as a prognostic factor in an increasing number of solid tumours. The nuclear DNA content can be assessed by denisitometric image cytometry DNA analysis. Both PCNA and DNA analysis can be performed on histological sections from paraffin embedded biopsies. In search of efficient and reproducible methods to identify early glottic cancers with increased risk for recurrence after radiotherapy, the PCNA expression as well as the DNA content of the diagnostic biopsies from 28 T1N0M0 glottic cancers were assessed. The group of tumours which recurred locally after radiotherapy displayed lower PCNA expression and higher DNA aberration than the group of tumours which were cured. Moreover, a combination of both parameters improved the possibility to discriminate the two groups. For T1 glottic cancer displaying high grade of genetic instability or low grade of proliferation, treatment regimes other than radiotherapy and closer follow-ups could be considered.
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INTRODUCTION

SQUAMOUS CELL carcinoma of the glottic larynx constitutes a significant proportion of head and neck cancers. The majority of patients with tumours diagnosed at an early stage are cured by radiotherapy, but patients with recurrent or persisting disease frequently cause diagnostic and therapeutic problems. Conventional histopathological grading is subjective, shows poor reproducibility, and gives at best a crude approximation of tumour cell kinetics and biological behaviour. In order to increase the prognostic value of histopathology for head and neck cancers, malignancy grading systems have been applied [1]. These systems, relating morphological findings to clinical outcome, provide additional information, but remain subjective, and the reproducibility is still unsatisfactory [2]. Methods of identifying the tumours with relative radioresistance would enable us to identify the patients who could benefit from alternative treatment and more frequent follow-ups.

Proliferating cell nuclear antigen (PCNA) is a nuclear protein involved in DNA replication, acting as the auxiliary protein of DNA polymerase δ [3, 4]. PCNA has proved to be a useful proliferation marker [5]. Quite recently, a series of monoclonal antibodies to PCNA has been described [6] including PC10, which is commercially available. PC 10 can be used on paraffin sections by immunohistochemistry, thus providing not only an environmental advantage compared with conventional radioligand binding quantitation but also spatial correlation permitting detection of small populations of marker positive cells.

Aberration in nuclear DNA content indicates genetic instability of the tumour. Image cytometry DNA analysis offers a quantitative, objective and reproducible method for assessing nuclear DNA content of a tumour cell population with preserved histopathology.

There is no previous report on the combination of nuclear DNA content and PCNA expression and its capability of predicting tumour recurrence after radiotherapy in uniformly treated T1 glottic cancer.

MATERIALS AND METHODS

Diagnostic biopsy specimens from 28 patients with squamous cell carcinoma of the glottic larynx (T1N0M0, according to UICC) were investigated (Table 1). There were 13 consecutive patients with recurrent ($n = 10$) or persistent ($n = 3$) disease, henceforth connoted as recurrent tumours, and 15 age matched randomly selected patients cured after radiotherapy during the same period of time.

There was 1 woman and 27 men; median age was 60.5, range 39–76 years of age. All patients were treated with

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Table 1. Clinical details for each patient: sex (male = m, female = f), age, grade of histopathological differentiation (well, moderately, poorly), %5c exceeding rate, PCNA positive rate, and recurrence or not

Patient no.	Sex	Age	Grade of diff.	%5cER	PCNA pos rate	Recurrence
1	m	43	Moderately	30	0.077	Yes
2	m	69	Well	17	0.500	Yes
3	m	73	Well	7	0.938	Yes
4	m	47	Poorly	47	1.000	Yes
5	m	45	Poorly	40	0.680	Yes
6	m	76	Poorly	29	0.759	Yes
7	m	60	Well	13	0.714	Yes
8	m	59	Moderately	7	1.000	No
9	m	47	Poorly	20	0.714	No
10	m	55	Poorly	3	0.750	No
11	m	61	Moderately	13	0.722	No
12	f	76	Moderately	3	1.000	No
13	m	67	Moderately	20	0.800	No
14	m	68	Moderately	3	1.000	Yes
15	m	66	Well	17	0.562	No
16	m	67	Well	0	1.000	No
17	m	66	Moderately	0	1.000	No
18	m	66	Well	4	0.939	No
19	m	62	Moderately	2	0.500	Yes
20	m	61	Poorly	4	0.727	Yes
21	m	61	Moderately	21	0.833	No
22	m	59	Well	3	1.000	No
23	m	57	Poorly	32	1.000	Yes
24	m	55	Moderately	0	0.667	Yes
25	m	55	Moderately	17	1.000	No
26	m	53	Moderately	27	1.000	Yes
27	m	46	Moderately	7	1.000	No
28	m	39	Moderately	1	1.000	No

mega-voltage irradiation, either with ^{60}Co or 4 MV linear accelerator. Two anterior oblique wedged fields 6 × 7 cm were used. A total dose of 64 Gy was delivered in fractions of 2 Gy, 5 days a week (CRE > 17.8). The follow-up time was at least 36 months. Median time to recurrence was 8 months, range 1–34 months. 4 patients died due to their cancer disease.

From the paraffin-embedded biopsy specimens, 4 μm thick serial sections were cut. The first section was stained with haematoxylin–eosin. The second section was immunohistochemically stained for PCNA analysis [6]. The section was deparaffinised in xylene and passed through a graded ethanol series. It was fixed in acetone for 5 min and incubated with 1% NP40 for 5 min. After blocking with 0.5% H_2O_2 in water and 1% bovine serum albumin (BSA) in Tris buffered saline (TBS) the murin monoclonal antibody PC10 (Novocastra, Newcastle upon Tyne, U.K.) was added and the section incubated over night at 4°C in humified chamber. After washing, the slide was incubated with biotinylated anti-mouse IgG. A third layer of ABC Vectastain Kit Elite and DAB was added. Finally a weak counterstain to visualise the nuclei was used. The third section was stained according to the Feulgen technique for image cytometry DNA analysis [7].

The histopathological specimens were reviewed by JL. There were seven well differentiated, 14 moderately differentiated, and seven poorly differentiated tumours.

For the PCNA analysis, tumour cells were selected systematically at random at 400 × magnification (light microscope, Olympus BH-2) using a point grid (100 points per field of vision) overlaying the microscopic image [8]. Ten fields of vision per specimen were analysed. Normal basal cells of the squamous epithelium were used as controls. The ratio between PCNA-ab stained and total amount of tumour-nuclei analysed was used to quantify the PCNA positivity of the individual tumour.

For the DNA analysis, a densitometric device—the Ahrens image analysis system (Bargtheide, Hamburg, Germany) was used. The DNA content of 100 tumour cell-nuclei per specimen was registered. Lymphocytes were used as control cells to establish the normal, diploid, 2c value. DNA content between 2c and 4c is seen in premitotic cell-nuclei, but cell-nuclei with DNA values exceeding 5c are not normally found in healthy squamous cell epithelium, and thus considered as aneuploid. The number of tumour cells with nuclear DNA content exceeding 5c was expressed in per cent, and was referred to as 5c exceeding rate (5cER) to quantify the degree of DNA aberration.

Statistical methods

Wilcoxon–Mann–Whitney test was used to investigate if (a) the rate of PCNA stained cells and (b) %5cER, differed between the recurrent and the cured group of tumours.

Fisher's exact test was used to investigate the power to discriminate the recurrent and the cured group with a combination of PCNA positive rate and %5cER.

Spearman rank correlation was used to analyse any significant correlation between (a) grade of histological differentiation and %5cER, (b) grade of histological differentiation and risk for recurrence, and (c) PCNA positive rate and %5cER.

RESULTS

The group of T1 glottic cancers which recurred after radiotherapy displayed a lower rate of PCNA positive cells than the non-recurrent group ($P = 0.040$, Wilcoxon–Mann–Witney test) Fig. 1.

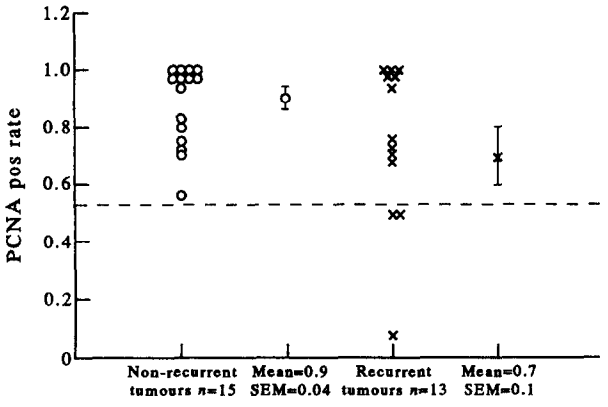

Fig. 1. The group of tumours which recurred (×) after radiotherapy displayed a lower rate of PCNA positive cells than the non-recurrent (○) group ($P = 0.040$, Wilcoxon–Mann–Whitney test).

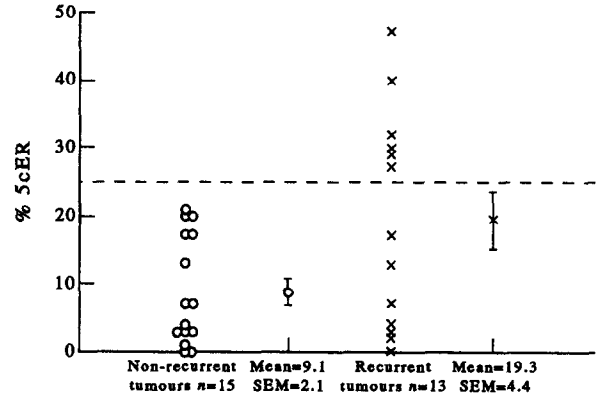

Fig. 2. The DNA aberration was more pronounced in the recurrent (×) group of tumours compared with the cured (○) group ($P = 0.047$, Wilcoxon–Mann–Whitney test).

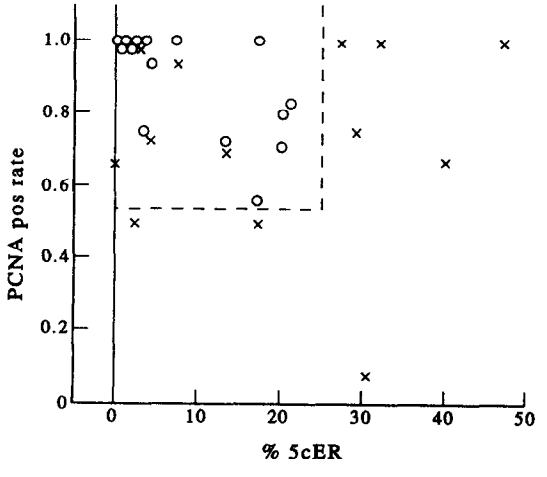

Fig. 3. By combining the PCNA and the DNA analysis, and using the cut off levels >0.5 PCNA positive rate, and <25%5cER all the non-recurrent (○) tumours and 8/13 (62%) of the recurrent (×) tumours were correctly classified $P = 0.004$, Fisher's exact test).

The DNA aberration was more pronounced in the recurrent group of tumours as compared with the cured group ($P = 0.047$, Wilcoxon–Mann–Witney test) Fig. 2.

By combining the PCNA and the DNA analysis and using the cut off levels >0.5 PCNA positive rate and <25%5cER, all the non-recurrent tumours and 8/13 (62%) of the recurrent tumours were correctly classified ($P = 0.004$, Fisher's exact test) Fig. 3.

Median 5cER was 7% for both well and moderately differentiated tumours, while poorly differentiated tumours displayed a median 5cER of 29%. No statistically significant correlation was found in %5cER between the three grades of histological differentiation (Rho = 0.4, $P = 0.054$).

Recurrence was found in three out of seven well differentiated tumours, five of 14 moderately differentiated tumours, and five of seven poorly differentiated tumours. No significant correlation between recurrence and grade of histological differentiation could be revealed (Rho = 0.02, $P = 0.293$).

No significant correlation between %5cER and the rate of PCNA positive cells was found (Rho = 0.01, $P = 0.329$).

DISCUSSION

At the Karolinska Hospital, early glottic cancer is, with few exceptions, primarily treated with radiotherapy giving a high cure rate and good preservation of the voice. In the case of tumour persistance or recurrence, salvage surgery is performed. However, a persisting or recurrent tumour may be concealed, for instance by post-radiation oedema, and thus become fatal in spite of repeated laryngoscopies and biopsies. If, the relatively radioresistent tumours could be recognised by methods of analysis of the diagnostic biopsy, the choice of treatment could be individualised and optimised.

In an earlier report from our hospital, 177 consecutive patients with cancer *in situ* and T1 glottice cancer were reviewed. Treatment failure after primary radiotherapy was analysed in detail, but could not be attributed to treatment irregularities [9]. No difference in pretreatment tumour size was detected when cured patients were compared with patients who relapsed. Biological factors causing relative radioresistance were considered to be the main reason for therapy failure in early glottic cancer.

The degree of cellular proliferation within tumours is believed to give a fair appraisal of their degree of biological aggression. Fast growing cancers are generally more rapidly fatal than slowly growing ones. In several types of cancer, the proliferation rate has been found to elicite prognostic significance [10]. Chauvel *et al.* using the thymidine labelling method, found that patients with head and neck cancers with a high labelling index (LI) had a significantly lower survival than patients with a lower LI [11]. From another point of view, however, the effects of radio- and chemotherapy can be expected to be better in a tumour cell population with a high proliferation rate, since cycling cells are a prerequisite for such antitumoral therapy to be effective.

Tennvall and co-workers found a lower proportion of cells in S-phase in the group of T3 laryngeal cancer which recurred after radiotherapy compared with the non-recurrent group [12]. Silvestrini and co-workers did not find pretreatment LI to be indicative of response to radiotherapy for oral cavity cancers, whereas a significant decrease in LI induced by 10 Gy radiotherapy implied a better chance for disease free survival [13].

The fraction of S-phase cells determined by flow cytometry DNA histogram analysis gives reproducible results in a majority of cases, but aneuploidy and cell debris can make the analysis less accurate. Furthermore, resting (G0) cells cannot be discriminated from cycling G1 cells. The use of monoclonal antibodies to cellular antigens associated with proliferation has contributed a relatively simple and reproducible method for studying the degree of cellular proliferation. In cultures of human epidermal keratinocytes, a general concordance between PCNA immunoreactivity and autoradiographically demonstrated thymidine incorporation has been found [5]. Furthermore, the PCNA-PC10 immunostaining technique maintains cellular and tissue architecture, permitting visual identification of the analysed tumour cells in a histological section. This is important, especially in epidermoid tumours, which may contain large hypoxic regions with slowly or non-cycling cells. The present study revealed that the group of T1 tumours cured by radiotherapy displayed a higher degree of proliferation, as reflected by PCNA positive rate, than the recurrent group.

Previous DNA analyses of head and neck cancer have shown quite divergent results, as far as frequency of aneuploidy, prognostic value, and the power to predict radiosensitivity are concerned. The frequency of aneuploidy in laryngeal cancer ranges from Walter's 17% ($n=24$ T1 tumours), via Cooke's 45% ($n=60$, 50 of these were T3T4 tumours) to Holm's and Böcking's series where all laryngeal cancers were found to harbour aneuploid cells [14–17]. These divergent results can be attributed to different methods of analysis and classification criteria. Cells with DNA content exceeding 5c are aneuploid (some possibly polyploid). However, we prefer, when performing DNA measurements on histological sections, not to classify the *tumours* as diploid or aneuploid, but rather to use the per cent 5c exceeding rate (%5cER) which gives a quantitative estimation of DNA aberration. In our series of 28 T1 glottic cancer, 3 of the cancer specimens lacked cells with DNA content exceeding 5c, whereas all other tumours displayed aneuploid cells to various degrees.

Franzén *et al.* found that non-diploid head and neck cancers responded better to radiotherapy than diploid cancers [18]. In a later study of T1 and small T2 glottic tumours, submitted to radiotherapy, the same group demonstrated that 5 out of 22 (23%) of the non-recurrent and 6 out of 11 (55%) of the recurrent tumours were non-diploid [19]. This is in agreement with Walter's series of 29 T1 laryngeal cancers, where all five aneuploid T1 laryngeal cancers occurred in the radioresistant group of tumours [14]. In the present study, the group of recurrent tumours exhibited a higher degree of DNA aberration than the non-recurrent group. None of the non-recurrent tumours, but 6 out of 13 in the recurrent group displayed 5cER above 25%.

Ensley found a strong direct correlation between degree of DNA aneuploidy and S-phase fraction for squamous cell carcinoma of the head and neck [20]. Chen, however, found that the DNA index correlated to degree of differentiation and to malignancy grade, while S-phase was related to mitoses alone [21]. S-phase calculations made from flow DNA histograms generally show a stronger correlation to degree of DNA aberration as compared with growth fraction estimates conducted with specific antibodies against proliferation antigens [22, 23]. This implies that the results of the former may be influenced by the degree of aneuploidy in the tumour. It may thus be better to use separate analyses to achieve more specific estimates for proliferative activity and genetic instability, respectively. In the present study, where the single cell image cytometry method was used for DNA analysis and immunostaining with mab PC10 was used for PCNA analysis, no significant correlation between % 5cER and PCNA positive rate could be found.

The proliferative activity of a tumour cell population may have different implications. For tumours treated with surgery alone a high proliferation rate, generally a fast growing tumour, ought to be a negative prognostic sign. On the other hand, a high proliferation rate in tumours treated with radio- or chemo-therapy would be expected to mean a good response to therapy. The genetic instability of a tumour cell population, would however generally be a bad prognostic sign, indicating a high malignancy potential as well as ability to select for radio- as well as chemo-therapy resistant clones. We found that a combination of PCNA and DNA analysis increased our ability to discriminate between the recurrent group of tumours and the non-recurrent group. All the non-recurrent and 62% of the recurrent tumours were correctly classified. There is a need for other complementary modes of analysis in order to predict radiosensitivity and recurrence risk for individual tumours. This could help us to optimise treatment modality and follow-up for these patients.

Several investigators have found a correlation between DNA ploidy and degree of histological differentiation for head and neck cancer, i.e. an increase of non-diploid/aneuploid tumours as degree of histological differentiation decreases [21, 24]. Other reports contradict this finding, thus implying that nuclear DNA content is a variable unrelated to histological grade [25–26]. This investigation found no significant correlation between the three degrees of histological differentiation and DNA aberration (%5cER). However, the poorly differentiated tumours showed a higher %5cER than the well and moderately differentiated tumours.

The present study revealed no significant correlation between recurrence rate and degree of histological differentiation. This is in accordance with earlier findings in a large series of T1 glottic cancer as well as our own experience from studies on T1 tongue cancer [9, 27]. These findings emphasise the need for other objective and reproducible parameters to characterise the biological behaviour of these tumours.

Although the majority of patients harbouring T1 glottic cancer are cured by radiotherapy, methods capable of identifying the tumours at risk of recurrence are needed. Conventional histopathology shows severe limitations in this respect. The present investigation showed that the group of T1 glottic cancers which recurred after radiotherapy displayed a lower degree of proliferation, as reflected by PCNA positive rate, or higher degree of genetic instability, as reflected by DNA aberration, than the non-recurrent group. By combining the PCNA and DNA parameters, the discrimination between the two groups was enhanced. The clinical consequence of these findings, if confirmed by ongoing prospective studies, would be alternative modes of treatment and more intense follow-up for patients harbouring T1 glottic cancer with a low PCNA positive rate or high DNA aberration.

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