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PCNA - a Cell Proliferation Marker in Vocal Cord Cancer. Part II: Recurrence in Malignant Laryngeal Lesions

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Abstract. *Laryngeal squamous cell carcinoma constitutes the most frequent carcinoma found in the head and neck region. A precise prediction for recurrence potential cannot be done on site, treatment and histologic grading. Since Proliferating Cell Nuclear Antigen (PCNA) and DNA-cytometry have shown a good correlation between premalignant lesions and their progressive potential towards full-fledged carcinoma in the larynx as described in part I of this work, we have analyzed the PCNA index and DNA cytometry in specimen taken from vocal chord carcinomas with a 5-year follow-up, in order to assess its relationship with the presence or absence of tumour progression. 42 cases with (21) and without (21) recurrence have been examined. The DNA-index ranged from 1.01 to 1.43 (mean 1.10) in the group without and from 1.02 to 1.59 (mean 1.38) in the group with recurrent carcinoma (p=0.002). The PCNA-index ranged from 0.00% to 18.90% (mean 6.97%) in the non-recurrent group and from 0.00 to 3g.50% (mean 16.35%) in the patients with recurence (p=0.001). Both indices also correlated in a highly significant way. From these data emerges a highly significant correlation between the cytometric indices of cell proliferation and PCNA immunostaining. Furthermore the high correlation between PCNA and DNA-index is of special interest for single case assessment. High DNA aberration and PCNA-index in vocal chord carcinoma may indicate a higher cellular aggressiveness of the tumour, resulting in a greater overall risk of metastases and local recurrences. Our results support the thesis that the indices of cellular proliferation within some cancers can define subsets of patients of high risk and help in isolating a population in which a more aggressive clinical protocol may be proposed.*

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Laryngeal squamous cell carcinoma accounts for about 2% of all newly diagnosed diseases in the Western World, and constitutes the most frequent carcinoma found in the head and neck region (1)(2).

It is known that clinical tumour progression in laryngeal squamous cell carcinoma (LSCC) is highly dependent on anatomical site, but in cases with similar gross morphology TNM classification (3) and surgical treatment are equally important. Some tumours recur and undergo metastasis resulting in the death of the patient, while others are cured (4). A precise prediction cannot be made from the site and treatment alone. However, up to now histologic grading has not shown a clear correlation with prognosis, and remains highly subjective (5).

A clear marker for the potential of progressive disease and recurrence risk remains unavailable, but there is increasing evidence that some proliferation markers can give helpful information (6) (7) (8) (9). Tumour proliferative activity is an important factor in the prediction of its further biological behaviour, and can be a guide in the choice of correct treatment. Therefore information about cell kinetics together with histological classification may help in the assessment of prognosis (10) (11) (12).

Among others, Proliferating Cell Nuclear Antigen (PCNA) has shown a good correlation between premalignant lesions and the progressive potential of full-fledged carcinoma in the larynx (13). PCNA is a 46kDalton acidic, non-histone, nuclear protein required for DNA synthesis, and acts as the auxiliary protein of DNA-polymerase delta (14). Several studies

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Table I. *DNA and PCNA index correlation with recurrence of carcinoma.*

	Without Recurrence	With Recurrence	p
DNA-Index	1.10	1.38	0.002
PCNA-Index	6.97%	16.35%	0.001
Total examined cases	21	21	

Table II. *DNA aberration with and without recurrence.*

DNA aberration	Without Recurrence	With Recurrence
low	18	7
high	3	14
Total examined cases	21	21

[χ^2=9.882, p=0.002]

carried out using monoclonal PCNA antibodies have shown that PCNA accumulates in the cell nuclei during the S-phase and can be considered as a proliferation marker (15).

Besides this, in recent years cell proliferation determined by DNA-cytometry has been shown to be a significant prognostic factor for several human malignancies (16). The amount of DNA change in a proliferating tumour cell reflects its genetic instability and has been implicated as a prognostic factor in an increasing number of solid tumours (17). In head and neck tumours the DNA aneuploidy rates range from 50 to 70% (18), and some studies have suggested a correlation between recurrence rates and decrease in disease free intervals, with aneuploidy (19)(20).

The positive results of the PCNA marker in precancerous tissue *versus* full fledged carcinoma of the vocul chord as described in our previous work, suggested that we should extend our studies to established carcinomas. In this study we analysed the PCNA index of specimens taken from vocal chord carcinomas resected in our department, and whose 5-year follow-up showed the presence or absence of tumour progression. Furthermore, we have correlated the PCNA-index with DNA cytometry analysis of the S-phase.

Material and Methods

a) *Specimens.* We considered 42 cases of $T_1N_0M_0$ vocal chord carcinoma which were treated at our department with subperichondral chordectomy and which had a full 5 year clinical follow-up. All patients were treated with standard subperichondral chordectomy without radiotherapy. Grading was done for all specimens. 21 of these patients showed no recurrence, local or distant metastasis, or other signs of persistent disease and were considered cured, while in the other 21 tumour progression could be seen, with local recurrence and/or metastasis.

b) *Immunohistochemical staining for PCNA.* Paraffin sections 4 μm thick from the same blocks used for histological diagnosis, were air-dried overnight at room temperature and immunostained with the monoclonal antibody PC10 (Mab PC10)(21) at a dilution of 1:200, using the immunoperoxidase technique (ABC complex) with light hematoxilin counterstaining. All immunostained sections were examined using a ×100 objective. A minimum of 1000 cells in random fields were counted for each case. The PCNA index was defined as the number of cells with strong unequivocal nuclear staining, corresponding to cells in the S phase and divided by the total number of cells counted, expressed as percentage.

c) *Nuclear cytometry (densitometry).* DNA content was measured with a Zeiss VIDAS image analyzer. The integrated optical density of the nuclei was estimated on 5-micron thick histologic sections and then correlated with the density of the control diploid nuclei (tissue lymphocytes). This correlation, defined as the DNA-index, was equal to 1 when the tissue under investigation was made up of a cell population with a normal 2C DNA content (diploid), and tend towards 1.5 when a marked percentage of 3C (triploid) cells were present (in the S phase). We used Feulgen's method for staining, which is based on the interaction between Schiff's reagent and the aldehyde groups of the desoxyribose molecules, previously unmasked by acid hydrolysis (5N Hcl at 22°C for 60 minutes) which removes the purinic bases. In each section we examined 200-250 cell nuclei and 50 lymphocytes as control. Nuclei that appeared to be overlapping or were not clearly defined were excluded from the study.

d) *Statistical analysis.* The difference between the mean DNA- and PCNA-index values in the two groups was calculated by variance analysis (F-test). The linear correlation between the two proliferative indices was determined using the Spearman rank test. The selected level of significance was $p<0.05$.

Results

Standard histologic grading did not reveal any significant correlation with recurrence in the cases examined by us.

The DNA-index ranged from 1.01 to 1.43 with a mean value of 1.10 in the group without recurrence, while in the patients with recurrent carcinoma the values were from 1.02 to 1.59 (mean 1.38%), with a statistically significant difference (p=0,002).

The PCNA-index ranged from 0.00% to 18.90% (mean 6.97%) in the non-recurrent group and from 0.00 to 38.50% (mean 16.35%) in the patients with recurrence. The level of statistical significance was p=0.001. (Table I).

Furthermore, the DNA-index and PCNA-index correlated in a highly significant way, both in the cases without (ANOVA, correlation index=0,737, $p<0.001$) and with, recurrence (ANOVA, correlation index=0.762, $p<0.001$) (Figure 1).

Considering DNA aberration alone(22), we found 17 cases classified "high aberration" and 25 cases with "low aberration". In the first group 14 cases (82.3%) had recurrence, while this occured in only 7 cases (28.0%) of the second group. Statistical analysis with the χ^2-test confirmed the significance of this data (χ^2=9.882, p=0.002) (Table II).

Discussion

The degree of cellular proliferation in neoplastic tissue is

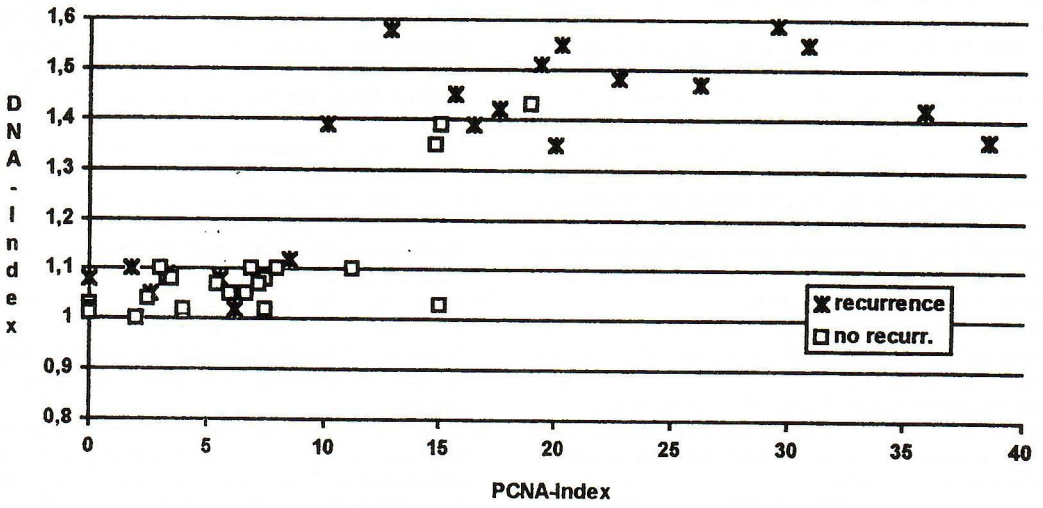

Figure 1. *DNA vs PCNA Index in cases with and without recurrence.*

generally agreed on as a good marker for the degree of biological aggressiveness. Fast-growing cancers are more rapidly fatal than slow-growing ones, thus the proliferation rate may have prognostic significance (23). A higher proliferation rate implies a larger group of cells in the S-phase with a con-comitant rise in the risk of further disarrangements of the genome and abnormal cellular DNA content as a reflection of chromosomal instability.

While distant metastases are linked not only to the degree of dedifferentiation and genetic disarrangement of the neoplastic cell, dependent largely on local factors such as lymphatic drainage, local recurrences are also an excellent model of correlating the true neoplastic cell behaviour and its genetic parameters. The poor lymphatic drainage of vocal chord carcinoma and its isolated location make it behave, at least for the first phases of its growth, as a local malignancy, closely resembling plant neoplasms (24) (25) (26) (27) (28) (29) (30) (31) (32) (33) (34) (35) (36). This neoplasm can be considered, as a good human model of locally invasive tumours (37).

Vocal chord carcinoma is a well - defined clinical entity with a fairly good response to treatment and prognosis (38). The very poor or absent lymphatic drainage in the true vocal chord explains the low degree af distant metastases and makes it an excellent model for the study of local invasiveness and cell behaviour. Local recurrences are rare but perhaps possible and we have collected 21 cases (LR) of these, as compared with 21 patients without recurrence (NR), in order to assess whether histologic indexes of dedifferentiation can help in predicting the local aggressiveness of the tumour.

Standard histologic grading has been considered a marker for tumour progression in many types of neoplatic tissue and the majority of LR's showed a high degree of DNA aberration and a high proliferation rate, as well as an intense PCNA positivity.

Furthermore our data showed a highly significant correlation between the cytometric indexes of cell proliferation and PCNA immunostaining, confirming the data reported by several other studies (39)(40).

Perhaps also if the overall statistics are highly significant for each group, the presence of "outsiders" such as low PCNA and DNA-index values in patients with recurrence (one WR with PCNA=0) should be kept in mind. Isolated values are still not consistent enough to determine clinical treatment in the individual patient. For this reason the high correlation between PCNA and DNA-index gains a special interest. The correlation between both factors and recurrence rises our understanding of cancer in the individual. Our results support the theory that the indices of cellular proliferation within some cancers can define subsets of patients with high risk.

To conclude, our data shows that high DNA aberration and PCNA-index in vocal chord carcinoma may indicate a higher degree of cellular aggressiveness in the tumour, resulting in a greater overall risk of metastases and local recurrence. These parameters can help in isolating the high risk population for whom a more aggressive clinical protocol would be advisable.

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