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Nucleolar organizer regions in laryngeal cancer

The conventional TNM classification system has been generally accepted and it provides useful prognostic information, but it is now well known that the biological aggressiveness of a solid tumour cannot be evaluated only on the basis of standard clinical and pathological parameters. Recent reports from the literature demonstrate that the evaluation of the quantity of interphase AgNORs is an independent prognostic factor in several types of human tumours (7, 15).

Nucleolar organizer regions (NORs) are chromosomal segments in which ribosomal RNA is encoded and they are responsible for the development of the RNA-containing nucleolus or nucleoli (12). The number of interphase AgNORs is strictly related to rRNA transcriptional activity and, in continuously proliferating cells, to the rapidity of cell proliferation (6). NORs can be visualized as black dots by a simple silver staining technique.

In laryngeal cancer, some authors found a significant prognostic correlation of the quantity of Argyrophilic Nucleolar Organizer Regions (AgNORs), whereas others did not. Therefore, it seems interesting to define the prognostic value of AgNORs count per nucleus in relation to the known clinico-pathological features and survival rates of patients with laryngeal carcinoma.

MATERIAL AND METHODS

The tissue for diagnostic specimens was obtained from 48 patients (46 males and 2 females) with laryngeal carcinoma who underwent either primary surgery, radiotherapy or combined therapy for tumours between 1991 and 1993. The patients were treated in the Department of Otolaryngology, Medical University of Lublin. Age ranged from 26 to 78 years (mean age 56 years). All patients included in the study were followed-up for at least 3 years or until death. Patients who died from causes other than laryngeal cancer were excluded from the study. The TNM cancer classification system was based on the 1987 UICC standards.

In paraffin-embedded samples from 48 patients with laryngeal cancer, staining and counting were performed according to the method described by P l o t o n et al. (11). Tissue sections were cut into 3-μm thicknesses, dewaxed in xylene and rehydrated through an alcohol series to deionized water. Slides were incubated with 1% dithiothreitol aqueous solution for 30 min at room temperature and washed off with deionized water. They were then exposed in the dark place for 35 min at room temperature to a freshly prepared staining solution made up of one part of 2 g/100 ml gelatin in 1%

formic acid solution and two parts of 50% aqueous silver nitrate solution. After washing with deionized water, sections were dehydrated through graded ethanols to xylene and mounted in histological mounting medium. AgNORs were counted according to the recommendations of C r o c k e r et al. (4), by which each AgNORs is seen separately within the nucleolus and is included in the count together with AgNORs outside the nucleolus. The dots of AgNORs were counted using Jenamed 2 (Karl Zeiss Jena) microscope (ocular magnification x 40). In the process of counting, the semi-automatic computer system of image analysis was applied (MultiScan, Poland). In each specimen, random fields containing 100 nuclei were examined. When AgNORs were in the form of clusters they were counted as a single dot.

The nonparametric chi-square test was applied for statistical analysis of the correlation between AgNORs score and clinico-pathological factors as well as survival rate. The value of $p<0.05$ was considered to be stastistically significant.

RESULTS

The results of the AgNORs counts and the clinico-pathological data for the present series of laryngeal carcinoma are summarized in Table 1:

Table 1. Clinico-pathological findings and AgNORs number in laryngeal cancer

Clin.- pathol. features	AgNORs no. (mean ± SD)	Total		p
		n	%	
Clinical stage I	2.86 1.03	8	16.6	NS
Clinical stage II	2.85 1.02	6	12.5	
Clinical stage III	2.66 0.66	19	39.6	
Clinical stage IV	2.71 1.02	15	31.3	
G 1	2.26 0.66	10	20.8	p<0.05
G 2	2.67 0.83	26	54.2	
G 3	3.27 0.88	12	25.0	
N 0	2.64 0.86	28	58.3	NS
N 1	2.81 0.85	15	31.3	
N 2	3.22 1.17	4	8.3	
N 3	2.39 0.00	1	2.1	
Survival rate < 5	2.77 0.95	23	47.9	NS
Survival rate=>5	2.70 0.81	25	52.1	

The positive staining of AgNORs was obtained in all cases of laryngeal cancer. Mean AgNORs counts were 2.73 ± 0.87 (range 1.48 – 4.38).

The mean AgNORs number in various clinical stages of disease (from I to IV) was similar and differences between them were not statistically significant. Likewise no significant difference in the AgNORs counts in patients with present and absent metastatic changes in regional lymph nodes was observed.

In the studied group of patients the significant difference in the AgNORs counts in the patients with G1, G2 and G3 stage of histological differentiation of carcinoma was observed ($p<0.05$).

In the present study, a statistically significant correlation between AgNORs counts and patient survival rate was not observed.

DISCUSSION

A one-step silver colloid method (AgNOR staining) to demonstrate proteins associated with NORs was devised by Howell and Black (9) and Ploton et al. (11). NORs are considered to have some regulatory function in controlling the transcription of the genes for ribosomal RNA. Therefore, it would be expected that they might reflect synthetic or metabolic activity of cells (5). Although AgNORs are reported to be a prognostic marker of laryngeal cancer cellular proliferative activity the findings are still controversial. In the present study, the AgNORs counts were determined in 48 primary laryngeal carcinomas and correlated with the most important clinico-pathological parameters as well as with survival rates. The average AgNOR number for squamous cell carcinomas in this study was smaller than in other reports (1, 2, 10, 13). This may be attributed to many factors, including section thickness, staining reaction time and convention for counting of AgNORs.

In the investigated group of patients the significant difference in the AgNORs counts in the patients with G1, G2 and G3 stage of histological differentiation of carcinoma was observed ($p<0.05$). By studying the correlation between AgNOR counts and histopathologic findings, it was found that AgNOR counts showed a lower value in the histologically low grade tumours (grades I and II), and higher value in high grade ones (grade III). This result is compatibile with the findings of Kręcicki et al., who found a significant correlation between AgNORs number per cell and tumour histological grading (10). Also, Yamamoto et al. (14) observed that tumours with poor histological differentiation tended to have more AgNORs, but the difference was insignificant. Hirsch et al. (8), Cappiello et al.(2) did not reveal a statistically significant correlation between the AgNORs count and the stage of histological differentiation.

The results of the present analysis failed to show any statistically significant correlation between AgNOR counts and lymph node status, clinical stages of disease as well as patient survival rate. This result is compatibile with Cappiello et al.(2), who also found no correlation of AgNORs counts with T category and presence of neck node metastases. In contrast, the prognostic relevance of AgNOR numbers in the laryngeal carcinoma has been reported by Bockmühl et al. (1), who used multivariate analysis to show that this parameter behaved as an independent variable in predicting survival. Kręcicki et al. (10) documented a significant correlation between the mean AgNORs number and survival rate in univariate and multivariate analysis, but they did not find a significant correlation between AgNORs count and T stage and lymph node metastasis. In the series of 66 squamous cell carcinomas of the head and neck Hirsch et al. (8) demonstrated a significant correlation between the mean AgNOR number per cell and tumour stage suggesting that AgNOR number is related to the biological aggressiveness of malignant squamous tumours. The prognostic significance of AgNORs in 69 patients with squamous carcinomas of the larynx was assessed by Xie et al.(13).

Considering the mean number of AgNORs, the authors revealed significant differences between carcinomas in patients with T1-2 compared with T3-4 tumours and significant lower mAgNORs scores in non-failures than in failures.

The lack of prognostic value of mean AgNORs numbers in this study might be explained in two ways. Firstly, the mean AgNOR number might not reflect the total rRNA gene activity in laryngeal cell carcinomas. Secondly, although cellular differentiation and proliferation have been related to AgNOR activity (3), there may be AgNOR-unrelated factors which also govern the growth and/or spread of laryngeal cell carcinomas. It appears that AgNOR enumeration has a limited prognostic value and further studies are necessary on larger groups of patients to assess its usefulness. However, the results of this study suggest that AgNORs counts may reflect the degree of malignancy and may be the helpful research and diagnostic tools in the evaluation of the biological activity of neoplasms.

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SUMMARY

The purpose of this study was to estimate the prognostic value of the AgNORs count per nucleus in laryngeal cancer. The sections of diagnostic, formalin-fixed and paraffin-embedded specimens from 48 patients with T1-4 tumours were stained with silver nitrate for visualization of NORs (AgNORs). The correlation between AgNORs score and tumour clinicopathological features was estimated. The mean AgNOR number per nucleus was 2.73 ± 0.87 (range 1.48-4.38). The AgNOR counts did not correlate with the stage of the disease, patient survival rate and N stage. A significant correlation between the mean AgNORs number and various stages of histological differentiation of carcinoma was observed ($p<0.05$).

Regiony organizatorów jąderkowych w raku krtani

Celem przeprowadzonych badań była ocena wartości prognostycznej liczby AgNORs w raku krtani. Materiał do badania stanowiła tkanka raka krtani, pobrana w celach diagnostycznych od 48 pacjentów. Pobrane wycinki były utrwalane w formalinie i zatapiane w bloczkach parafinowych. W celu wizualizacji obszarów organizatorów jąderkowych skrawki były poddawane reakcji wysrebrzania przy użyciu azotanu srebra. Oceniano korelację pomiędzy liczbą AgNORs a stopniem klinicznego zaawansowania raka krtani, obecnością przerzutów do regionalnych węzłów chłonnych, stopniem histologicznego zróżnicowania guza oraz okresem przeżycia pacjentów. Średnia liczba ziarnistości AgNOR przypadająca na jąderko wynosiła 2,73 ±0,87. Stwierdzono istotną statystycznie zależność pomiędzy liczbą AgNORs a stopniem zróżnicowania histologicznego guza. Nie stwierdzono istotnych zależności pomiędzy liczbą AgNORs a stopniem klinicznego zaawansowania raka krtani, obecnością przerzutów do regionalnych węzłów chłonnych oraz okresem przeżycia pacjentów.