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Nucleolar Organizer Regions (NORs) as a prognostic factor in patients with squamous–cell oesophageal cancer

Prognostyczne znaczenie badania ekspresji regionów organizatorów jąderkowych (AgNOR) u chorych z płaskonabłonkowym rakiem przełyku

The proliferative activity of cells is a morphological indicator of their functional state. Morphological changes in the nucleolus are a permanent feature of neoplastic cells. The changes consist in the enlargement of the nucleolus and its less regular shape, compared with normal cells (5, 6). In malignant lesions AgNORs are often variously dispersed, which permits an evaluation of the proliferative activity with such parameters as: the average number and the total area of AgNORs, the mean area of a single AgNOR in a cell nucleus (13, 17, 19).

The qualitative evaluation of AgNORs has been considered a good marker of the proliferative activity of various neoplasms, including the squamous cell oesophageal carcinoma (5, 9, 10, 13, 15, 17, 18).

A nucleolus is a well defined functional–structural unit with no membrane, where ribosomes are produced and ribosomal genes are transcribed (10, 25). Chromosome segments are a significant element of the nucleolar structure. They contain large loops of DNA which codes for ribosomal RNA (rRNA) (10, 13, 19). The segments are referred to as nucleolar organiser regions.

In the karyotype of a human diploid cell NORs are located on the short arms of the chromosomes: 13, 13, 14, 21 and 22 (6, 19, 20). Silver–stained NORs are defined as AgNORs and silver–absorptive NOR proteins – as AgNOR proteins (5, 10, 20). It should be pointed out that the method primarily identifies acidic, silver–absorptive proteins associated with the areas of RNA transcription

(11). A biochemical definition of those proteins has not yet been formulated. It is believed that RNA polymerase I and proteins B23, C23 are stained during the NOR detection (6, 20). C23, also called nucleolin or protein 100 kDa, is the major silver–stained protein (6). The role of RNA polymerase I is to transcribe the rRNA of the ribosome precursor. Thus the number of AgNORs is thought to reflect the intensity of nuclear activity (20).

The aim of the present study was to determine whether the clinical features of the tumor, the depth of infiltration, status of regional and extraregional lymph nodes and the length of patient survival are related to the number of AgNORs in biopsy specimens taken before the operation from squamous cell oesophageal carcinoma.

MATERIAL AND METHODS

An analysis was carried out of biopsy material obtained from 60 patients with squamous cell carcinoma of the thoracic oesophagus who were operated on in the Surgery Clinic II of the Medical School in Lublin over the period of 1992–1995. The study involved 54 (90%) men and 6 (10%) women (9:1 ratio), aged from 43 to 70 (57.9 ± 8.0 age mean). On the basis of radiologic, endoscopic (in selected cases – EUS – endoscopic ultrasonography), ultrasonographic (USG) and computer tomography (CT) findings the clinical stage of the neoplasm was defined after the UICC classification from 1987 (8). Except for 10 cases, the patients were given to alcohol abuse and an ingrained habit of smoking. Forty patients from the group had undergone chemo– or chemo– and radiotherapy before the operation.

Histopathological examination was carried out in the Department of Pathomorphology of the Medical School in Lublin supervised by Prof. Daniel Chibowski. Both the endoscopic and the operative specimens were fixed in 10% buffered formalin for not more than 24 hours (biopsy specimens – for about 6 hours). In each case 3 to 6 endoscopic biopsy from oesophageal tumor were obtained. Operative specimens were opened longitudinally and 3 to 8 full thickness blocks of the tumor as well as surgical margins and lymph nodes of appropriate groups were routinely taken and embedded in paraffin wax. 4 μm sections were cut and routinely stained with hematoxylin and eosin (H&E) and mucicarmine. The depth of invasion (pT), the number of examined lymph nodes, including the number of nodes with metastasis and their distribution (pN), the presence of remote metastasis in extraregional lymph nodes (M_{lym+}) and, thereby, the pathological stage of the cancer (pTNM) were defined in the study. The size of the lesion, the macroscopic pattern of the tumor and oesophageal vessels invasion were also examined.

AgNORs were visualized using Lindler's silver–staining technique (14): specimens were incubated in a mixture of one volume of 2% gelatin in 1% formic acid and two volumes of 50% silver nitrate. Incubation was performed for 30 minutes at the room temperature in a dark place.

The counting of AgNORs was performed in representative areas of the specimens at 400x magnification. In each case one hundred neoplastic nuclei were analyzed and the mean number of AgNORs per nucleus was estimated. The material was divided into two groups – cases with the mean of AgNORs count per nucleus <2 and these with count >2.

METHODS

A number of statistical methods were used to show the relations and differences relating to the problems of the study. Differences in the mean values were assessed with T–student test. Cases of death resulting from post–operative complications (post–operative mortality) were excluded from the analysis. Patients were observed for 2 to 25 months after they had been discharged from the hospital. Survival curves were constructed using the Kaplan–Meier method. The effect of the analyzed parameters on survival was estimated with the Cox's proportional risk model, while their influence on the occurrence of a specified phenomenon was measured with the logistic regression model since few of the parameters displayed normal distribution. Many non–parametric tests were used in the analysis, including the Kruskal–Wallis test and Sperman and Kendal's non–parametric correlation. Results were considered statistically significant if $pH < 0.05$.

RESULTS

The study revealed that in 7 (11.6%) cases the lesion was located in the upper third of the thoracic oesophagus, in 40 (66%) cases – in the middle, and in 13 (21.6%) cases – in the lower part of the oesophagus. Radiological examination was used in defining the type of the lesion. The most frequent one – the funneled type – was found in 25 (41.7%) patients, the spiral type in 21 (35%) patients, the serrated type – in 10 (16.7%) patients, and the tumors type – in 4 (6.7%) patients. In the endoscopic image the lesion in 23 (38.3%) cases was defined as diffuse–infiltrating, in 19 (31.7%) cases – as ulcerating and infiltrating, in 10 (16.7%) cases as prominent, and in 8 (13.3%) cases – as ulcerating and non–infiltrating. The length of the lesions, evaluated through computer tomography, ranged from 30 to 120 mm (62 ± 21.0) and their width – from 15 to 26 mm (17.3 mm on average). On the basis of the TNM classification the carcinoma stage in 19 (13.6%) patients was defined as IIa, and in the remaining 41 (68.3%) patients – as stage III.

Morphological findings in surgically treated patients are presented in Table 1. It emerges that in 8 (13.3%) cases the carcinoma was diagnosed as well differentiated, in 29 (48.3%) cases – as moderately differentiated, and in 18 (30%) patients – as poorly differentiated. In 5 cases no neoplastic cells were found in the operative specimen – pT0. They were the patients who had undergone chemotherapy and in whom a full response to or a mixed effect of the treatment were obtained. In 2 (3.3%) cases the lesion was confined to submucosa (pT1), in 10 (16.6%) cases it comprised the muscle layer (pT2), in 25 (41.6%) cases it infiltrated the whole wall (pT3), and in 18 (30%) cases – oesophagus–adjacent tissues. Lymph node metastasis was found in 34 (56.6%) patients – in other 26 (43.3%) cases no neoplastic cells were identified. Moreover, in 11 (18.3%) cases extraregional lymph nodes revealed metastasis. Among patients who had fully responded to preliminary treatment in 3 (5%) cases the carcinoma was classified as stage 0, in 2 (3.3%) cases – as stage I, in 15 (25%) cases – as stage IIa, in 3 (5%) cases – as stage IIb, in 26 (43.3%) cases – as stage III, in 11 (18.3%) cases – as stage IV. Vessel invasion occurred in 33 (55%) cases.

Table 1 shows the basic clinical and pathomorphological findings related to the number of AgNORs. AgNORs were seen as black or dark brown dots within the nucleus. Sometimes they formed larger clusters. They were present in the nuclei of all cells. In carcinoma cell nuclei AgNORs were heterogeneous in number, shape and distribution. Fibroblasts, lymphocytes, macrophages or endothelial cells contained 1 to 2 granules.

Tab. 1. The AgNOR number in biopsy specimens (n=60) and selected clinical and morphological findings

	<2	>2	p
Sex			ns
1 – male	16 (94.1%)	38 (88.4%)	
2 – female	1 (5.9%)	5 (11.6%)	
Age	60.2 ± 8.6 44 – 70	56.9 ± 7.6 43 – 70	ns
Smoking			ns
1 – non smoker	3 (17.6%)	7 (16.3%)	
2 – up to 20 cigarettes a day	12 (70.6%)	27 (62.8%)	
3 – over 20 cigarettes a day	2 (11.8%)	9 (20.9%)	
Cancer location			ns
2 – upper section	0	7 (16.3%)	
3 – middle section	13 (76.5%)	27 (62.8%)	
4 – lower section	4 (23.5%)	9 (20.9%)	
Metastasis in extraregional lymph nodes (pM_{lym})			ns
pM_{lym-}	9 (82.9%)	20 (80%)	
pM_{lym+}	6 (17.1%)	5 (20%)	
Histological differentiation			ns
1 – no neoplastic cells	3 (17.6%)	2 (4.7%)	
2 – highly differentiated squamous cell cancer	4 (23.5%)	4 (9.3%)	
3 – moderately differentiated sq. cell cancer	6 (35.3%)	23 (53.5%)	
4 – poorly differentiated sq. cell cancer	4 (23.5%)	14 (32.6%)	
Depth of the lesion – pT			p<0.05
pT0	3 (17.6%)	2 (4.7%)	
pT1	1 (5.9%)	1 (2.3%)	
pT2	3 (17.6%)	7 (16.3%)	
pT3	8 (47.1%)	17 (39.5%)	
pT4	2 (11.8%)	16 (37.2%)	
Lymph nodes evaluation – pN			p<0.0005
pN0	16 (94.1%)	10 (23.3%)	
pN1	1 (5.9%)	33 (76.7%)	
Cancer stage – pTNM			p<0.0005
0	3 (17.6%)	0	
I	1 (5.9%)	1 (2.3%)	
II a	9 (52.9%)	6 (14.0%)	
II b	0	3 (7.0%)	
III	3 (17.6%)	23 (53.5%)	
IV	1 (5.9%)	10 (23.3%)	
Vessel invasion			p<0.02
1 – yes	5 (29.4%)	28 (65.1%)	
2 – no	12 (70.6%)	15 (34.9%)	

The case of patients with postoperative clinical metastasis that occurred within six months led to the observation that before the operation 22 (91.7%) of them had been characterized as having a high (>2) AgNOR number, and 2 (8.3%) – as those with a low (<2) number of AgNORs. This relationship was statistically significant ($p<0.001$).

The relation between the number of AgNORs with regard to the pT, pN, pM_{lym+}, markers and selected tumor features clinically assessed before the operation has been illustrated in Table 2. The Table demonstrates that the number of AgNORs correlated with such markers as pT, pN and pTNM ($p<0.0005$). While no significant connection was observed between extraregional lymph node metastasis and the number of AgNORs, the relation between the invasion of vessels in the oesophageal wall and the number of AgNORs proved relevant ($p<0.02$).

A high number of AgNORs was significantly more frequent in the diffuse–infiltrating and the ulcerating–infiltrating lesion types, defined on the basis of the previously mentioned endoscopic assessment. The length of the tumor correlated with a high AgNOR number ($p<0.002$, $p<0.001$), which both the CT and the radiological examinations testified to. No relation was found between the values of the examined markers and such parameters as: age, sex, neoplasm location and the degree of carcinoma differentiation.

Tab. 2. Correlation between the AgNOR number and selected features of the tumor – preoperative estimate

	p
Tumour length – CT evaluation	$p<0.02$
Width of the lesion – radiology	n.s.
Length of the lesion – radiology	$p<0.001$
Macroscopic image – endoscopic evaluation*	$p<0.01$ typ: 4, 3, 2, 1

Macroscopic image of the neoplastic lesion in endoscopic evaluation: 1) prominent type, 2) ulcerating and non infiltrating type, 3) ulcerating and infiltrating type, 4) diffuse – infiltrating type. The way the types have been mentioned responds to the decreasing value of the examined marker.

Prognostic (survival length) values of the marker analyzed in a biopsy specimen (before the operation) are shown in Cox's model of proportional risk was applied in constructing the chart. There was a statistically significant difference ($p<0.0003$) between the survival of patients with carcinoma characterized by a low AgNOR number (<2) and the survival of those with a high AgNOR number (>2). Patients with a low AgNOR number were likely to survive longer.

DISCUSSION

It is commonly known that morphologic changes in the nucleolus are a fairly permanent feature of neoplastic cells. Malignant cells reveal a larger and less regular nucleolus than benign cells (6) A quantitative evaluation of the fact seems to be of primary diagnostic and prognostic importance in carcinoma pathology (10, 11, 17, 20, 25). The evaluation has its basis in the number of AgNOR granules/clusters/ visible in the area of a cell nucleus.

Morita et al: (16) examined 98 cases of squamous cell oesophageal carcinoma obtained by endoscopic biopsy. The number of AgNORs in all cases was 4.93 ± 1.49 on average. The number was higher where the size of the tumor surpassed 5 cm. It was also strictly related to the depth of the lesion in the oesophageal wall, to blood vessel invasion and to lymph node metastasis. The authors did not discover any statistical difference for such features as sex, age or tumor location. Patients were divided into three groups, depending on the AgNOR number: those with a high (>6), medium (4 to 6) and low (<4) AgNOR number. The division was significant as patients with the AgNOR number <4 displayed the highest 5–year survival rate which accounted for 44.8% of cases. In other two groups the survival rate was: 38% – for the AgNOR number between 4 and 6, and 11% – for the number of AgNORs >6. The research they carried out made the authors conclude that the number of AgNORs in a preoperative oesophageal biopsy is a good indicator of the "malignancy potential" of the esophageal carcinoma and can serve as a marker in "selecting" patients for various therapy types, the combined treatment among them.

An examination of 91 patients with squamous cell oesophageal carcinoma carried out by the same authors in 1993 confirmed the earlier findings (17) The researchers adopted the same criteria of dividing patients into groups according to the number of AgNORs. They also applied cytophotometric analysis to define the DNA content (17) They acknowledged that a preoperative determination of the AgNOR number or the DNA content, in oesophageal biopsy specimens may be regarded as a good prognostic marker in the carcinoma. If the two methods are used simultaneously, the obtained prognosis is more reliable.

In the study carried out by the present authors, where the obtained values of the examined parameter were the basis of division, two groups of patients were formed: those with a low (<2) and a high (>2) number of AgNORs.

The number of AgNORs in oesophageal carcinoma as quoted by other authors was usually higher (10, 13, 16, 17, 18). The discrepancies may result from a different interpretation of the AgNOR image. It is assumed that twenty AgNOR spots should be visible in the nucleus of a human diploid cell where DNA had been synthesized (the G2 phase) (23). Immediately after mitosis the nucleus may contain 10 AgNORs which blend fast and form one nucleolus (21). In a normal cell AgNORs are tightly packed in nucleoli which are easily discernible in histologic preparations, while particular regions may often remain invisible (21). In neoplastic cells AgNORs are variously dispersed, which facilitates counting them (5). Therefore a quantitative estimate largely depends on the degree of the dispersion or disintegration of a relatively large number of AgNORs in the nucleus. It seems that their number reflects the numerical index of dispersion rather than the absolute number of regions (21). The AgNOR number can indicate not only their dispersion, but the transcriptional activity of the nucleus as well (5).

An increase in the number of nucleolar organisers may be related to a high proliferative activity of the cell, a connection defect in particular organisers, or augmentation of cellular ploidy (23). It should be pointed out, however, that interphase AgNORs may cluster or overlap, which makes the method poorly repeatable (6). There are two ways of approaching the AgNOR count. Firstly, all silver–stained structures may be counted. If they form groups, each cluster is counted as a single structure. Secondly, where AgNORs in the nucleolus are separated, each AgNOR may be counted as a separate entity together with the smaller organisers located and visualized outside the nucleolus (4). Accurate visualization and correct counting of nucleolar organisers are essential. Appropriate reaction period and binding methods should also be applied in the AgNOR detection technique (4). Conventional 10% formalin is generally a good method of fixing. Alcoholic fixatives can allow more intense

staining, yet they do not influence the number of AgNORs (4). In formalin methylene bonds between adjacent sulphydate and carboxyl groups are formed, which inhibits the silver staining of AgNORs. The bonds are broken when the tissue is washed in 70% alcohol. As a result, active groups become accessible for silver ions again (3). In the first studies on the number of AgNORs a 60–minute reaction was allowed. Recently shorter (approximately 30 minute) reaction periods have been applied (4).

In spite of difficulties with AgNOR evaluation it is evident from the research conducted so far that the number of interphase AgNORs in malignant cells is statistically larger than in nuclei of benign lesions (2, 4, 5, 6). That may testify to an increased metabolic activity in nucleoli, resulting from a larger number of ribosomes in proliferating cells (6). It has indeed been demonstrated that the number of interphase AgNORs depends on the speed of cellular replication. The greater the speed, the larger the number of interphase nucleolar organisers (6). The presence of AgNORs in the nuclei of benign lesions, where their number may be similar to that in malignant lesions, excludes the possibility of treating them as the only criterion of histologic malignancy. Where the neoplasm is permeated with lymphocytes and macrophages, there is no certainty that only neoplastic cell AgNORs are being counted (19).

Our analysis of preoperative AgNOR counts in patients with advanced oesophageal carcinoma revealed a statistically significant relation with respect to the depth of the oesophageal wall lesion ($p<0.05$), the carcinoma stage and the frequency of oesophageal vessel invasion ($p<0.02$). A statistically significant relation was also found between the image of the neoplasm (endoscopic evaluation) and the AgNOR number ($p<0.01$). Carcinomas infiltrating the oesophageal wall were characterized by a larger value of the AgNOR number. The findings concerning the depth of the lesion were consistent with Yoshida's observations (26). In their study of 96 cases of squamous cell oesophageal carcinoma the authors discovered statistically significant differences in the number of AgNORs relating to the depth of the carcinoma invasion ($p=0.02$), vessel invasion ($p=0.01$) and the carcinoma stage (pTNM) ($p=0.02$). No statistically significant differences were found between the AgNOR number and the patients' age, sex or carcinoma differentiation.

Other Japanese researchers came to different conclusions after they had assessed the AgNOR number in a group of 45 patients with advanced oesophageal carcinoma (markers T2, T3). They found no statistically significant difference in a given parameter with respect to the carcinoma differentiation degree, the depth of invasion or the vessel permeation (10).

A valuable observation was made by numerous researchers dealing with carcinoma metastasis. They found that in some carcinoma groups (squamous cell oral, pharyngeal and oesophageal carcinomas, as well as pulmonary, gastric, hepatic and mammary ones) an increase in the number of AgNORs is correlated with a high risk of neoplasm metastasis (6, 10, 17, 20). The problem seems important considering the influence of the lymphatic system permeation on patients' survival length (1). As we have mentioned, there are a number of image examinations and established rules of evaluating lymph nodes with respect to metastasis (the mere fact of nodes' visualization, their size, shape, the radiation reduction factor), yet they have not come up to clinicians' expectations (7, 22, 24, 26). In the group of patients the present authors examined a correlation occurred between the number of AgNORs and metastasis in lymph nodes ($p<0.005$). The result may prove the value of the method and its usefulness in the evaluation of preoperative readiness of the oesophageal carcinoma to metastasize. The study confirms the fairly unanimous opinion of other researchers with regard to the role of AgNOR counting in defining the degree of lymphatic system permeation.

Several groups of Japanese researchers revealed the relation between metastasis in lymph nodes and the AgNOR number (16, 17, 25). Ikeguchi et al. (10), as well as their predecessors, found statistically significant differences in lymph node metastasis ($p<0.001$) between groups of patients

with advanced oesophageal carcinoma. In their other publication the authors called attention to the fact that the proliferative activity of neoplastic cells may be influenced to some degree by the suppressive activity of regional lymph nodes (9). They discovered lymph node metastasis in 53 out of the 95 examined patients. The AgNOR number in neoplastic tumors in patients with "positive lymph nodes" amounted to n=53, (6.1 ± 1.8) and was higher than in patients with no metastasis in lymph nodes – n=42, (3.8 ± 1.1, p=0.001). In 39 patients with the metastasis the AgNOR number values were observed to be lower in the nodes than in the primary tumor. The 5–year survival rate in that group was 23.7%. Where the number of AgNORs was higher in the nodes than in the primary focus (14 patients), in 11 cases the period of survival was shorter than 3 years.

Maesawa et al. (15), who assessed the AgNOR number in 39 patients with superficial oesophageal carcinoma, found no correlation between the AgNOR number and lymph node metastasis. On the other hand, a relapse of the carcinoma (in less than 3 years) was more frequent in patients with a high number of visualized AgNORs.

The discussed – often divergent – views of researchers on the value of AgNOR counting testify to the complexity of the problem. All authors dealing with the issue agree, though, that the higher the number of AgNORs found in a celi nucleus, the worse the prognosis is likely to be (10, 12, 13, 15, 18, 25).

The results of our study seem to confirm those views. Cox's proportional risk model was found to differentiate the length of survival at a statistically high level ($p<0.0003$). Although the findings of the present study on the role of the AgNOR number as determining the clinico–pathological parameters seem encouraging, with the existent state of knowledge their clinical value is reduced to an auxiliary application in dubious cases. The potential prognostic value of proliferative parameters is promising, yet further research is necessary to test their usefulness in clinical practice.

CONCLUSIONS

The evaluation of nucleolar organizer regions (NORs) can be useful for the detection of the depth of the lesion and presence of the metastasis in lymph nodes in squamous esophageal cancer.

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STRESZCZENIE

Ekspresję regionów organizatorów jąderkowych (AgNOR) analizowano z użyciem techniki srebrzenia w skrawkach tkankowych u 60 pacjentów z płaskonablonkowym rakiem przełyku. Stwierdzono, iż wyższa liczba AGNORs w jąderku korelowała z wyższym stopniem zaawansowania raka, jak również z długością zmiany w przełyku: w badaniu KT – $p<0,02$, w badaniu radiologicznym skopii rtg – $p<0,0001$, w endoskopii – $p<0,01$, głębokość nacieku – $p<0,05$, obecność przerzutów w węzłach chłonnych – $p<0,0005$, stopień zaawansowania – $p<0,0005$, inwazja naczyń krwionośnych – $p<0,02$. Zaobserwowano również znacząco dłuższy okres przeżycia u pacjentów z niską liczbą AgNORs (<2) w stosunku do osób, u których liczba ta była wyższa od 2 ($p<0,0003$).

Uzyskane wyniki badania w wycinkach biopsyjnych ekspresji AgNORs sugerują ich przydatność jako markera w ocenie stopnia zaawansowania i rokowania w płaskonabłonkowym raku przełyku.