

CELL KINETICS ANALYSIS OF SURGICALLY RESECTED NON-SMALL CELL CARCINOMA OF THE LUNG USING THE AgNOR SILVER STAIN

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Cell kinetics analysis of lung carcinoma using DNA flow cytometry has shown a significant correlation with the biological behavior of these neoplasms. Ploidy has shown a more significant association with aggressive behavior. The method may however not be available in all centers. Two counts of the AgNOR silver stain have been correlated with ploidy and proliferative activity (PA). The first count, which is the mean number of AgNOR granules (mAgNOR), correlates with ploidy. The second count is the percentage of cells with ≥ 5 AgNORs/nucleus (pAgNOR), reflects PA. We performed the AgNOR silver stain using the two above-mentioned counts in 41 cases of surgically resected non-small cell carcinoma of the lung. The cases included 14 adenocarcinomas, 24 squamous cell carcinomas, and three undifferentiated non-small cell carcinomas. Follow-up data were available on 36 of the patients, ranging from 10 to 31 months (median 18 months). Thirteen of these patients (36%) developed progressive disease. Adenocarcinomas showed mAgNOR counts suggestive of aneuploidy (≥ 2.4) in nine of the 14 patients (64%) and 16 of the 24 squamous carcinomas (66%). The adenocarcinomas showed high pAgNOR counts ($\geq 8\%$) in eight of the 14 cases (57%), in contrast to 15 of the 24 squamous carcinomas (62%). The AgNOR counts did not show any statistically significant correlation with tumor type, grade or stage of disease. The mAgNOR counts were aneuploid in all 13 progressive cases and in only 10 of the 23 stable cases (43%) ($P=0.001$). The pAgNOR counts were high in 12 of the 13 cases that progressed (92%), in contrast to 10 of the 23 stable cases (43%) ($P=0.01$). There is no significant evidence that squamous carcinoma of the lung may have a higher incidence of aneuploidy and high PA than adenocarcinoma. Our data also confirm previous data showing that aneuploid lung carcinomas have more aggressive behavior than diploid ones. This study also indicates that, despite the short-term follow-up data, the use of the AgNOR silver stain for cell kinetics analysis of non-small cell carcinoma of the lung may potentially provide useful predictive information on the biologic behavior of lung carcinoma. Long-term follow-up may provide more significant information. *Ann Saudi Med* 1997;17(2):161-166.

Lung carcinoma is the most common cause of cancer death in North America, killing approximately 200,000 people in 1993.¹ Despite aggressive and modern therapy, the overall prognosis of lung cancer has not improved much over the years.² Prognostic indicators have therefore been sought to identify subsets of tumors that would require more aggressive therapy. Cell kinetics analysis of the neoplasms are such predictors of aggressive behavior. The method of kinetic analysis most widely used is DNA flow cytometry.³ It has been shown that lung carcinomas exhibiting aneuploidy have more aggressive behavior than diploid ones.⁴⁻⁹ It has also been shown that tumors with high proliferative activity manifested by high S-phase fraction and S + G2M phase present at high stages of

disease.¹⁰ Flow cytometry unfortunately does not allow concomitant morphological assessment of the evaluated cells for comparison. The method is also not available in all centers.

The argyrophilic nucleolar organizer regions (AgNORs) are loops of DNA present on the short arms of acrocentric chromosomes (13, 14, 15, 21 and 22).¹¹ A silver staining technique was developed in the early seventies which enabled researchers to assess AgNORs in routinely processed tissue.¹² Interphase AgNOR has been shown to correlate with ploidy and/or proliferative activity.¹³ Distinction between ploidy and proliferative activity based on the AgNOR silver stain has been mainly achieved through the work of Mourad et al. using two AgNOR counts.^{14,15} It has been shown that the mean interphase AgNOR count (mAgNOR) correlates with ploidy.^{14,15} It has also been established that the AgNOR proliferative index (pAgNOR), which is the percentage of nuclei exhibiting ≥ 5 AgNORs/nucleus, correlates with proliferative activity.¹⁴⁻¹⁸

In the current study we attempted to correlate the above-mentioned AgNOR counts, as a function of ploidy and proliferative activity, with certain parameters in 41 cases of surgically resected non-small cell carcinoma of

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the lung. The parameters included tumor grade, type, disease stage and short-term prognosis.

Materials and Methods

Patient Population and Clinical Staging

Forty-one patients with the histological diagnosis of non-small cell carcinoma of the lung were the basis for this study. The cases were accrued consecutively. All patients had definitive surgical treatment in the form of wedge resection, lobectomy, or pneumonectomy. Most of the patients had preoperative mediastinoscopy, except for those with peripheral lesions with no evidence of mediastinal lymphadenopathy on CT scan. The patients were clinically staged according to the criteria established by Mountain.¹⁹

Pathological Evaluation

The resected tissue was assessed by one pathologist and tumor typing and grading was done following the criteria of the World Health Organization guidelines.^{20,21} Cases depicting a mixed pathological pattern were typed according to the predominant pattern. No case in our series fulfilled the criteria for a mixed carcinoma. Pathological staging was done following the guidelines of the International Union Against Cancer (UICC).²² The pathological stage (pTNM) was the final stage designation in our study.

Clinical Follow-Up

The patients were followed on a monthly basis for the first three postoperative months. A full physical examination and plain chest x-ray were performed every three months thereafter for three years. Additional laboratory investigations, such as biochemical profiles, complete blood counts or other radiological studies, were performed if the patients were symptomatic. Patients showing no evidence of tumor recurrence or metastatic disease had stable disease and those showing evidence of recurrence or metastatic disease were put in the category of progressive disease.

The AgNOR Silver Stain

Representative sections of the resected neoplasms were selected for the AgNOR silver staining technique. Four-micron unstained sections were obtained from these areas to perform the stain. The modified AgNOR silver staining technique introduced by Ploton et al. was used.¹² The tissue was deparaffinized in several changes of xylene and descending alcohol concentrations. Rehydration was then performed in several changes of ultrapure distilled water. The tissue was then incubated in acid alcohol (3 parts ethanol : 2 parts acetic acid) for five minutes and then rinsed in ultrapure distilled water several times. The sections were then incubated with silver nitrate solution in a dark humidified chamber for 38 minutes. The silver staining solution consisted of two parts

TABLE 1. Clinical and pathological data pertaining to the 41 patients studied and their corresponding AgNOR counts.

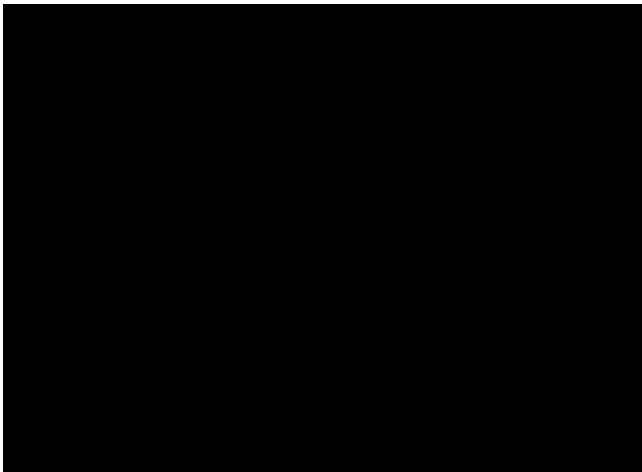
#	Age/sex	Tumor type/grade	Stage	F/U		mAgNOR	pAgNOR
				L	O		
1	67/M	ADENOCA III	3A	28	AND	1.88	6
2	56/F	ADENOCA III	1	15	AWD	3.60	25
3	67/F	ADENOCA II	1	27	AWD	3.10	14
4	56/M	ADENOCA II	1	31	AND	2.30	7
5	61/M	SCC I	1	27	AND	2.09	9
6	73/F	SCC II	1	19	DOD	3.48	12
7	62/M	UNSCC	1	15	AND	2.10	4
8	76/M	SCC II	3A	19	AND	2.28	7
9	75/M	ADENOCA II	3A	25	AND	1.54	2
10	70/M	SCC II	2	26	AND	1.41	0
11	75/M	ADENOCA II	3A	0*	DND	2.69	11
12	64/M	SCC III	1	0*	DND	3.65	25
13	64/M	ADENOCA III	1	27	AND	2.99	13
14	76/M	SCC II	2	25	AND	2.69	18
15	43/M	ADENOCA II	1	23	AND	3.75	25
16	74/M	SCC III	2	-	NO F/U	4.01	43
17	57/M	ADENOCA III	3A	25	AWD	2.63	19
18	54/M	SCC III	2	23	AWD	2.87	12
19	58/M	SCC III	2	10	AWD	2.47	1
20	82/M	SCC III	1	0*	DND	2.53	5
21	64/M	SCC II	2	23	AWD	3.63	18
22	49/F	ADENOCA III	3A	25	AND	2.46	7
23	53/F	ADENOCA III	2	24	AND	1.52	3
24	61/M	SCC III	2	18	AWD	3.01	18
25	48/M	UNSCC	2	14	AWD	3.02	15
26	70/F	SCC III	3A	19	AND	4.57	45
27	72/F	UNSCC	2	0*	DND	2.33	5
28	58/M	SCC III	2	19	AND	2.95	8
29	40/F	SCCIII	2	19	AWD	3.85	35
30	67/M	SCC III	3A	19	AND	3.23	23
31	75/F	SCC III	3B	19	AWD	3.01	16
32	75/M	ADENOCA I	2	18	AND	2.62	5
33	62/M	SCC III	2	22	AWD	2.99	12
34	69/M	SCC III	3A	13	AND	1.93	7
35	58/M	SCC II	3A	15	AND	1.63	5
36	45/M	ADENOCA III	2	15	AND	3.12	16
37	66/F	SCC II	2	15	AND	1.69	3
38	80/M	SCC III	2	13	AND	2.30	8
39	66/M	ADENOCA III	3A	16	AND	2.41	11
40	73/F	SCC III	4	11	AWD	3.11	20
41	67/M	SCC III	2	12	AND	1.49	1

M=male; F=female; F/U=follow-up; L=length; O=outcome; SCC=squamous cell carcinoma; ADENOCA=adenocarcinoma; UNSCLC=undifferentiated non-small cell carcinoma; AND=alive with no evidence of disease; AWD=alive with disease; DND=dead with no evidence of disease (autopsy); DOD=dead of disease.

of a 50% solution of silver nitrate and one part 2% gelatin in 1% formic acid solution. Ultrapure distilled water was used for preparation of all solutions. The sections were then incubated with a 10% solution of sodium thiosulfate solution for five minutes. The sections were then washed in distilled water, dehydrated in graded alcohol and then xylene and coverslipped. The tissue was then ready for counts. No counter stain was used.

AgNOR Counts

The AgNOR counts were performed by one investigator without knowledge of the tumor type, grade, stage, or disease



This analysis was performed with mAgNOR and pAgNOR in relation to tumor type, grade, disease stage and short-term outcome in terms of stability or progression of the disease.

Results

The 41 patients included 30 males and 13 females, with a male to female ratio of more than 2:1. The patients' ages ranged from 40 to 82 years with a median of 66 years. There were 24 cases of squamous cell carcinoma, 14 cases of adenocarcinoma, and three cases of undifferentiated non-small cell carcinoma of the lung (UNSCLC). The patients' combined clinical and pathological stages were as follows: Stage I (n=11), stage II (n=17), stage IIIA (n=11), Stage IIIB (n=1), and stage IV (n=1). Follow-up data were available on 36 of the 43 patients (83.7%). Four patients died in the immediate postoperative period and one patient was lost to follow-up. Follow-up periods ranged from eight to 31 months with a median of 18 months. Patients with follow-up periods of less than 12 months were those who developed recurrence, metastasis, or died within those periods. Of the 36 patients with available follow-up data, 13 (36%) developed progressive disease. This was mainly manifested by locoregional recurrence in 10 patients and metastases in two. One patient died of disseminated metastases 12 months postoperatively.

AgNOR Counts

Aneuploidy was indicated when tumors had mAgNOR counts of 2.4 or more. These counts were seen in 16 of 24 squamous cell carcinomas (66%), in nine of 14 adenocarcinomas (53%), and in one of three UNSCLC. Although the number of aneuploid squamous cell carcinomas was somewhat higher than that for adenocarcinoma, the association was not statistically significant. No association between mAgNOR and tumor grade or stage of the disease was seen.

pAgNOR counts were high ($\geq 8\%$) in 15 of the 24 cases of squamous cell carcinoma (62%), in 8 of the 14 cases of adenocarcinoma (57%) and in one of the three

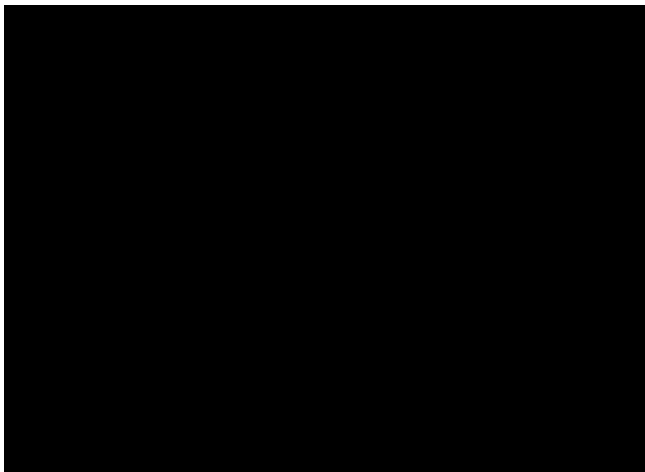
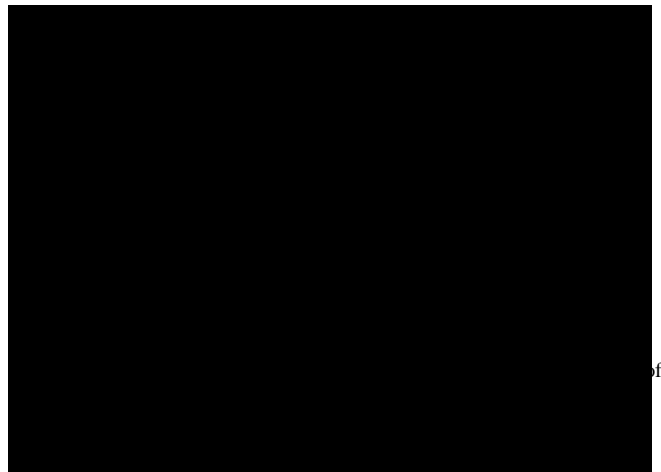
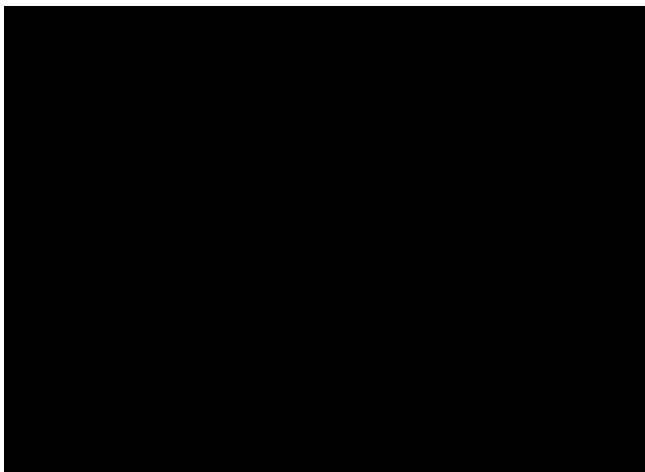
cases UNSCLC. In spite of the relatively higher incidence of increased proliferative activity in squamous cell carcinoma in comparison with adenocarcinoma, a statistically significant association was not observed.

AgNOR Counts in Relation to Short-term Disease Outcome

The 13 cases showing progressive disease all had aneuploid mAgNOR counts (100%). These counts were only seen in 10 of the 23 cases of stable disease (43%), which indicates a significant difference between the two groups ($P < 0.001$; Figure 1). Cases showing progressive disease had high pAgNOR counts ($\geq 8\%$) in 12 of the 13 cases (92%). Only ten of 23 cases with stable disease (43%) had pAgNOR counts above 8%. The difference was also statistically significant ($P < 0.01$; Figure 2). This difference was seen regardless of the tumors' grade or type. Some tumors were poorly differentiated but had low AgNOR counts (Figure 3), whereas others that had the same histological grade had high counts (Figure 4).

Discussion

The AgNOR silver staining technique has been extensively studied to find alternative techniques of cell kinetics analysis other than flow cytometry as a method. A method that would provide for the evaluation of both ploidy and proliferative activity of neoplasms and at the same time assess the morphology of these neoplasms would be the ideal choice. Most studies assessing the AgNOR silver stain proposed that interphase AgNOR reflects ploidy and/or proliferative activity.¹³ There has actually been a controversy about whether AgNOR really correlates with ploidy and/or proliferative activity. Some studies have concluded that AgNOR reflects proliferative activity and not ploidy.²⁴⁻²⁶ These studies were mainly using the correlation of interphase AgNOR counts with the known proliferation markers ki-67,²⁴⁻²⁶ bromodeoxyuridine (BrDU),²⁶ or the proliferating cell nuclear antigen (PCNA).^{27,28} Unfortunately, studies using the same markers concluded that AgNOR does not really reflect proliferative activity.²⁹⁻³¹ In these latter studies no statistically significant correlation could be obtained with those proliferation markers. Another study using AgNOR in trophoblastic disease has proposed that AgNOR truly reflects ploidy.³² It thus appears that it would be impossible to reconcile these opposing viewpoints. All the above-mentioned studies have been using one single AgNOR count. This count was the mean number of

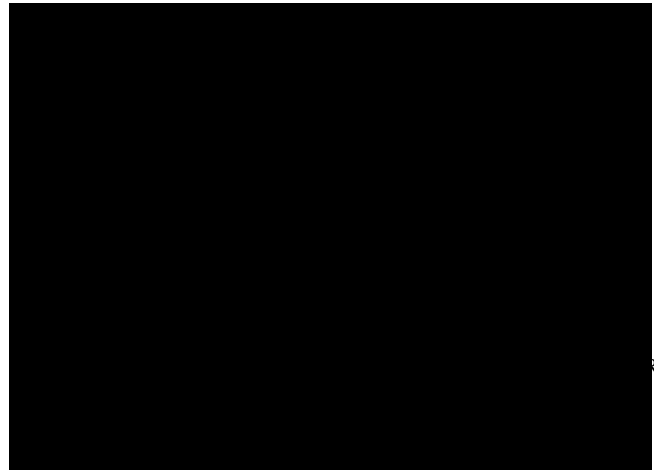


When we studied both interphase and metaphase AgNOR in Wilms' tumor,³³ we found that the number of interphase AgNORs is much smaller than those in metaphase. This finding was also seen in a study of interphase and metaphase AgNORs in malignant lymphoma.³⁴ This has led us to propose that interphase AgNORs are truly the reflection of the spatial arrangement of the AgNOR-carrying chromosomes and that the number of interphase AgNORs would be the reflection of cellular activity.¹⁴ Cells that are actively synthesizing DNA (S phase) or in the process of dividing (G2M phase) would have their chromosomes separated, giving a high interphase AgNOR count. We therefore proposed that mAgNOR would probably be the reflection of the total number of chromosomes or ploidy and the percentage of cells with

proliferative activity. We studied those two counts in breast carcinoma and compared them with ploidy and S-phase fraction (SPF) measured by flow cytometry and found a significant correlation.^{14,15} We also correlated the two counts with proliferative activity measured by BrDU and Ki-67 and found that pAgNOR correlates with proliferative activity measured by these methods while mAgNOR does not.^{16,17} We also found that pAgNOR is more predictive of aggressive behavior in node-negative breast carcinoma than mAgNOR.³⁵ Additionally, we found that both mAgNOR and pAgNOR are more predictive of invasive potential than histological assessment in ductal carcinoma in situ of the breast.³⁶

Studies using DNA flow cytometry in non-small cell carcinoma have shown that aneuploidy reflects poorly on

FIGURE 4A. Photomicrograph of grade III squamous cell carcinoma of the lung (H&E, 40x).



disease outcome.^{3-9,37,38} These studies have shown that aneuploidy indicates higher incidence of recurrence,⁷ poor short-term survival,⁸ and lower overall survival rates.⁶ Proliferative activity in non-small cell carcinoma of the lung has been less frequently reported in the literature.⁵ There are, however, studies that have shown that increased proliferative activity in terms of the SPF and the growth fraction manifested by the S + G2M phases have shown that high proliferative activity is associated with higher stages of the disease.⁵ Studies measuring proliferation activity using methods other than DNA flow cytometry, such as the proliferating cell nuclear antigen (PCNA) and the proliferation marker ki-67, have shown a good correlation between these markers and disease outcome.^{5,39} In those studies where both ploidy and proliferative activity data were available in non-small cell carcinoma of the lung, ploidy seems to have more significant impact on predicting survival than proliferative activity.^{3-9,37,38} Ploidy is not always the most important parameter in predicting malignant behavior in all organ systems. For instance, proliferative activity is more significant in predicting aggressive behavior in breast carcinoma than ploidy.⁴⁰⁻⁴² In other instances, ploidy has no bearing on the biological behavior of some neoplasms, especially thyroid tumors.⁴³ In our study we found that aneuploidy as detected by the AgNOR silver stain was more significant in predicting aggressive behavior than proliferative activity. We also have found that aneuploidy, as measured by the AgNOR silver stain, is seen in over two-thirds of the 43 cases studied. This finding is also corroborated in the literature by studies evaluating the DNA content of non-small cell carcinoma of the lung using flow cytometry.^{4,5,6,8} In short, our study confirms previous data concluding that aneuploidy is a more significant prognostic indicator in non-small cell carcinoma of the

of cell kinetics lies in the fact that it allows for the concomitant morphological analysis while evaluating cell kinetics.

We conclude that cell kinetics analysis of non-small cell carcinoma of the lung can be performed using the AgNOR silver staining technique. The method provides good correlation with short-term disease outcome. Long-term follow-up information may yet provide more significant information in terms of the predictive value of the AgNOR silver staining technique in lung cancer.

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