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Original article

Comparative Evaluation of Argyrophilic Nucleolar Organizer Regions Parameters in Benign and Malignant Breast Tumors

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SUMMARY

Aim. The aim of the paper was to evaluate the AgNOR parameters for the discrimination of benign from malignant breast tumors via a new approach - the total AgNOR area/nuclear area (TAA/NA).
Material and methods. Three groups, consisting of control (n = 14), benign (n = 18) and malignant (n = 28) participants were included in the study. The AgNOR staining technique was performed and both mean AgNOR number and TAA/NA ratio were evaluated.
Results. While the differences between the control and patient groups were statistically significant for AgNOR number ($p < 0.001$), it was not significant between the malignant group and the benign group for mean AgNOR number ($p > 0.05$). For the ratio of TAA/NA, the differences between the control and benign group ($p < 0.001$), control and malignant group ($p < 0.001$), and malignant and benign patient groups were significant. ($p < 0.05$).
Conclusion. We consider that the evaluation of the TAA/NA rate, when compared with the AgNOR number, can be more sensitive and useful tool for distinguishing benign from the malignant breast lesions.
Keywords: breast, breast cancer, nucleolar organizing regions, AgNOR

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INTRODUCTION

Breast cancer is the most common cancer in women and the second leading cause of cancer death (1). Therefore, the development of reliable and reproducible diagnostic tests for early diagnosis is very important.

Nucleolar organizing regions (NORs) are DNA sequence containing ribosomal RNA genes located in the nuclei of cells play an important role in protein synthesis. Thus, the numbers and appearance of NORs reflect the rate of cell proliferation depending on the rate of ribosome construction and protein synthesis (2).

It was reported that the number of AgNORs was higher in cancer cells than in normal cells and they reduced silver nitrate to metallic silver in the presence of formic acid. The AgNOR method is a good technique for discrimination of malignant lesion from the benign (3).

The number of AgNOR spots has been assessed until now in most studies. However, AgNOR speckles can be of different magnitudes for reasons such as the convergence of more than one, the overlap. Hence, we think that the evaluation of the total AgNOR area/nuclear area (TAA/NA) may be more effective than the AgNOR count (4).

The analysis is based on the measurement of the ratio of the stained areas to the entire area of the nucleus (AgNOR regions) in a cell nucleus. For this reason, as in other proliferation determinants, cells do not need to be in a group within a certain area, even a total of 50 cells that can be evaluated on different slides can be enough to produce results. In this way, our method will solve the problem of inadequate material as an important problem of fine needle aspiration biopsy (FNAB).

Apart from the AgNOR method, some other techniques indicative of cell proliferation include immunohistochemical detection of increases in the expression of markers such as PCNA (proliferation cell nuclear antigen), Ki-67 (5). Success rates of these methods are variable not only in terms of discrimination between malignant and benign lesions but also in their differential diagnosis of malignant lesions (5). These methods' working principles are based on the determination of the ratio of the number of stained, marked, or tumor cells located in a specific area on the preparation stained with the relevant method; therefore, the diagnosis cannot be achieved if the amount of cells in that area is insufficient, or if all of the cells are unstained or unmarked in that respective area.

In this study, the total TAA/NA values and AgNOR count of 50 cells from FNAB sample of breast were evaluated using a special computer program designed for this purpose (6). In this regard, we aimed to determine whether this method can be used to distinguish benign from malignant breast carcinomas and their subgroups, by finding a cut-off value for breast cancer. We investigated the usability potential of the method in diagnosis at the individual level. Thus, we think that unnecessary costs, time loss, and operations etc. can be avoided, too.

MATERIAL AND METHODS

Forty-six patients (18 benign and 28 malignant) were included in the study. The obtained materials from suspicious breast lesions by using the FNAB method were spread out and then fixed with methanol and were air-dried.

The slides were stained with silver and air dried. Then images of the cells were transferred to a computer and the TAA/NA values and mean AgNOR number for 50 cells were calculated for each nucleus using a special computer program as a follow-up (6).

The ratio of the total AgNOR area in each nucleus to the same nucleus area was used as a criterion of evaluation. The study protocol was approved by the Clinical Research Ethics Committee at the University of Erciyes School of Medicine (2013/193).

Statistical method

Statistical analysis was done using Statistical Package for Social Sciences (SPSS 22) packet program. The descriptive statistical methods, Kruskal-Wallis Test and Mann–Whitney U tests were used for comparison of the groups. MedCalc version 13 (2014) package program was used to calculate a cut-off value. Data were given as mean ± SD and $p < 0.05$ values were accepted as statistically significant.

RESULTS

Pathology results and control group values

From the pathology laboratory, the cancer diagnosis results of patients in addition to FNAB re-

sult (Tru-cut, lumpectomy, and mastectomy) were also taken. Thus, 4 different types of results were recorded. Pathology results are also summarized in Table 1 and 2. According to this, there were 18 benign, 12 malignant, 6 nondiagnostic, and 10 suspicious at the FNAB; 7 benign, 7 malignant, 1 nondiagnostic at Trucut, 2 benign, 11 malignant at lumpectomy, 1 benign and 10 malignant at mastectomy. According to the final pathology result (Final PR), which is the gold standard of these results, there were two groups consisting of 18 benign and 28 malignant individuals with breast lesions.

A control group of 14 people was included in the study as the third group. The AgNOR numbers and TAA/NA ratios of all groups are shown in Table 3.

Table 1. *Patients with mean TAA / NA value lower than the cut-off value*

N	FNAB PR	Tru-cut	Lumpectomy	Mastectomy	Final PR	TAA/NA
1	Suspicious	Benign			Benign	1.69
2	Suspicious	Benign			Benign	2.92
3	Benign				Benign	2.99
4	Malignant		Malignant	Malignant	Malignant	3.31
5	Suspicious		Malignant		Malignant	3.37
6	Benign				Benign	3.41
7	Non-diagnostic		Malignant		Malignant	3.58
8	Non-diagnostic			Malignant	Malignant	3.64
9	Benign				Benign	3.7
10	Benign			Benign	Benign	3.74
11	Benign	Malignan			Malignant	3.77
12	Suspicious		Malignant		Malignant	4.01
13	Benign				Benign	4.03
14	Benign	Benign			Benign	4.1
15	Benign				Benign	4.19
16	Benign		Benign		Benign	4.38
17	Suspicious	Benign			Benign	4.4
18	Suspicious	Benign	Malignant		Malignant	4.45
19	Benign				Benign	4.55
20	Suspicious	Benign			Benign	4.67
21	Malignant			Malignant	Malignant	4.8
22	Benign				Benign	4.83

FNAB PR: Fine needle aspiration biopsy pathology results

Table 2. *Patients with mean TAA/NA value greater than the cut-off value*

N	FNAB PR	Tru-cut	Lumpectomy	Mastectomy	Final PR	TAA/NA
1	Malignant			Malignant	Malignant	4.85
2	Malignant		Malignant		Malignant	4.87
3	Non-diagnostic	Non-diagnostic	Malignant		Malignant	4.89
4	Malignant				Malignant	4.98
5	Benign	Benign			Benign	5.05
6	Benign				Benign	5.09
7	Benign	Malignant		Malignant	Malignant	5.09
8	Suspicious			Malignant	Malignant	5.78
9	Benign				Benign	5.8
10	Suspicious		Benign	Malignant	Malignant	6.1
11	Suspicious		Malignant		Malignant	6.13
12	Benign				Benign	6.13
13	Benign			Malignant	Malignant	6.38
14	Malignant		Malignant		Malignant	6.66
15	Malignant	Malignant			Malignant	7.59
16	Benign	Malignant	Malignant		Malignant	7.9
17	Malignant			Malignant	Malignant	8.85
18	Non-diagnostic	Malignant			Malignant	8.98
19	Malignant				Malignant	9.31
20	Malignant				Malignant	9.38
21	Malignant	Malignant			Malignant	9.41
22	Non-diagnostic		Malignant	Malignant	Malignant	9.96
23	Non-diagnostic	Malignant			Malignant	11.15
24	Malignant				Malignant	14.09

FNAB PR: Fine needle aspiration biopsy pathology results

Table 3. *TAA/NA ratios and mean AgNOR numbers of individuals*

	Malignant group		Benign groups		Control group	
N	AgNOR number	TAA/NA rates	AgNOR number	TAA/NA rates	AgNOR number	TAA/NA rates
1	2.72	14.09	1.28	4.55	1.2	2.81
2	7.18	11.15	1.22	3.7	1.07	2.34
3	7.08	8.98	2.08	5.09	1.2	2.7
4	1.18	7.9	2.08	4.19	1.13	3.21
5	3.06	9.38	1.37	4.03	1.27	2.57
6	2.6	7.59	1.37	4.83	1.29	3.59
7	3.46	8.85	1.74	6.23	1.07	1.9
8	2	5.78	2.2	3.41	1	1.77
9	2.26	6.66	2.31	4.38	1	3.03
10	2.68	9.96	1.88	5.8	1	1.92
11	3.14	9.31	2.79	2.99	1	2.12
12	1.36	6.1	3.64	5.05	1	2.8
13	3.95	5.09	6.05	4.4	1	2.26
14	2.48	4.85	2.83	4.1	1	2.13
15	1.15	6.38	1.54	3.74		
16	1.46	4.89	1.86	4.67		
17	3.02	3.64	2.62	2.92		
18	1.7	3.58	2.3	1.69		
19	8.32	9.41				
20	2.22	4.87				
21	5.74	6.13				
22	2.74	4.01				
23	1.53	3.77				
24	1.42	3.37				
25	2.82	3.31				
26	2	4.98				
27	3.17	4.45				
28	2.36	4.8				
Group averages	3.03 ± 1.86	6.55 ± 2.73	2.29 ± 1.13	4.21 ± 1.07	1.09 ± 0.11	2.51 ± 0.54

Comparison of groups AgNOR numbers and TAA/NA ratios

TAA/NA ratios and mean AgNOR numbers of benign, malignant and control groups were summarized in Table 4 and the mean AgNOR number, and TAA/NA ratio of groups were compared.

As can be seen in the Figure 1a, a statistically significant difference was found between control and patient groups ($p < 0.001$) for AgNOR number; in spite of that, no statistically significant difference was found between the malignant and benign group for mean AgNOR number ($p > 0.05$)

The bar graph in Figure 1b shows that the differences between the control and benign group ($p < 0.001$), control and malignant group ($p < 0.001$), and malignant and benign patient groups for the ratio of TAA/NA are significant ($p < 0.05$).

Table 4. *TAA/NA ratios and mean AgNOR numbers of groups*

	Control	Benign	Malignant	P
TAA/NA ratios (Mean ±SD)	2.51 ± 0.54	4.21 ± 1.07		< 0.001
		4.21 ± 1.07	6.55 ± 2.73	< 0.05
	2.51 ± 0.54		6.55 ± 2.73	< 0.001
AgNOR number (Mean ±SD)		2.29 ± 1.13	3.03 ± 1.86	> 0.05
	1.09 ± 0.11	2.783 ± 1.642		< 0.001

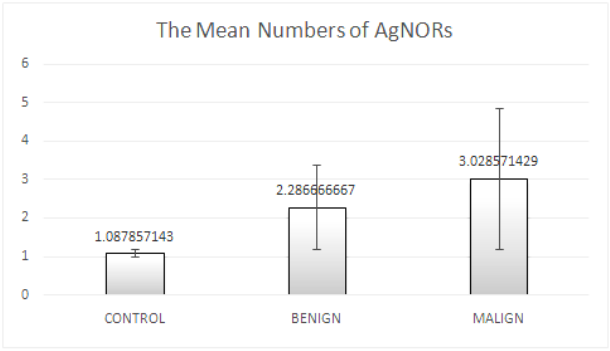

Figure 1a

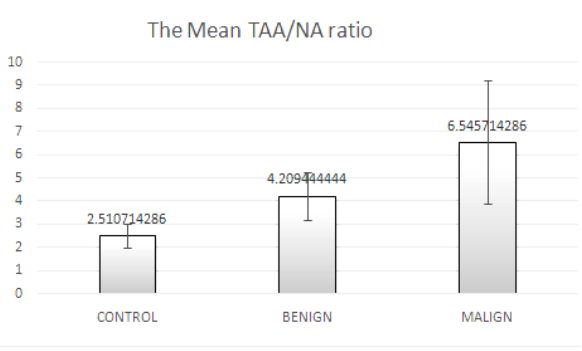

Figure 1b

Figure 1. *Mean AgNOR numbers (a) and the mean TAA/NA ratio values of the groups (b). The results of the measurements were plotted using individual mean values of the both AgNOR numbers and TAA/NA ratios, respectively were compared in this graphic.*

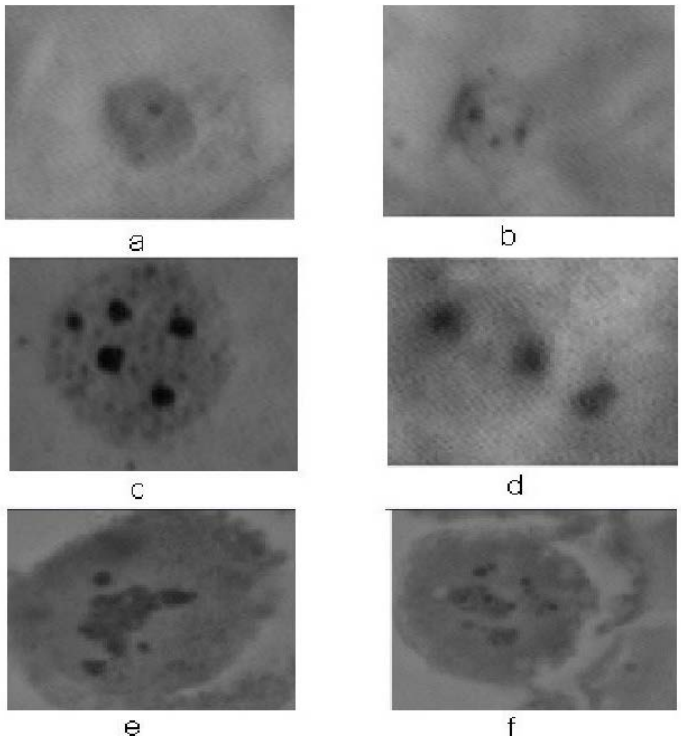

Figure 2. *View of cell nuclei by groups. As seen in this figure, both the AgNOR counts and TAA/NA ratio in the cell nuclei of the control group are lower than in the other groups. The distribution of AgNOR proteins in the nucleus is regular (a, b). In the benign patient's cell nuclei, both the AgNOR counts and the TAA/NA ratio are higher than the control group and lower than in the malignant patient group. Also, the distribution of AgNOR proteins in the nucleus is regular (c, d). As seen in Figure 2 (e, f), both the AgNOR counts and the TAA/NA ratio are higher in the cell nuclei of individuals who have received malignancy diagnosis than those in the control and benign groups. The distribution of their AgNOR proteins in the nucleus is also more irregular than in these groups.*

Finding the cut-off value and interpretation of results according to this value

According to Bayesian statistic results for discrimination benign from malignant lesion, the sensitivity (60.71%) and specificity (72.22%) were not statistically significant for mean AgNOR number ($p > 0.05$). However, the obtained specificity (77.78%) and sensitivity (71.43%) values via TAA/NA ratio to discriminate benign and malignant lesion were statistically significant ($p < 0.001$) (Table 5). To evaluate the results according to the obtained cut-off value, the TAA/NA averages values were ordered from the smallest to the largest in Table 1.

Table 2 shows the patients who are expected to be malignant according to the cut-off value.

A sample of control, benign and malignant group AgNOR count and TAA/NA ratio in the cell nuclei are shown in Figure 2.

DISCUSSION

Because the most common cancer type in women is breast cancer (1), development of early diagnostic methods that can be easily performed is much more important. Many studies have been performed which differentiate cancer cells from normal cells, based on the different spreading of interphase AgNOR proteins (2 - 4). According to these studies, interphase AgNOR proteins can be used to differentiate cancer cells from normal cells. However, quantitative evaluation of interphase AgNOR proteins has been proposed as a reliable tool to distinguish benign tumors from malignant tumors for only a few types of lesions (7).

Changes in the interphase AgNOR values have been shown to be associated with a rapid increase in tumor mass in patients with lung and liver cancer (8). The rate of tumor mass growth is one of the most important factors affecting clinical outcomes. The most efficient application of AgNOR parameters in tumor pathology is to describe the course of the disease. AgNOR values are monitored as an important parameter that provides information about the progression of the cancer disease. The presence of a significant correlation between the AgNOR value and the duration of the patient's survival has been described in several cancer types (2 - 8). The AgNOR staining method is applied to various neoplastic samples to distinguish benign and malignant lesions from pathological materials (4, 5, 7, 8). The number and distribution of AgNOR proteins among the cells with different proliferative activity showed statistically significant association among the cell proliferation determinants such as estrogen receptor protein, progesterone receptor protein, Ki-67, PCNA, c-erbB-2, mitotic index (9). However, the number of AgNORs cannot be as effective as the TAA/NA in terms of a cancer diagnosis. Instead of evaluating the number of AgNOR specks of different sizes, it would be more useful to measure the areas and compare them to the nucleus area.

According to our study, there is a significant difference between the mean AgNOR number of the control group and both the benign and malignant patient group. However, there is no statistically significant difference between the benign and the malignant group. When the TAA/NA ratio is taken into consideration, all groups are statistically different from each other. While the number of AgNOR cannot differentiate between benign and malignant lesions, the TAA/NA ratio can safely make this discrimination. As a matter of fact, the AgNOR number is criticized as an insecure method because these speckles can combine to form a single spot at any moment (10). In addition, the number of speckles can also change according to the person who count the AgNOR (11).

As for the literature review, some authors have argued that various AgNOR methods provide successful results for differential diagnosis (4, 12), while other authors have concluded that there may

be additional methods that fail or only aid the diagnosis (5, 13). In a study, it was reported that the prognostic value of the AgNOR parameter depends on the state of pRb and p53 tumor suppressor proteins and cannot be attributed to the relationship between AgNORs and cell proliferation rate. Whereas in another study of breast cancer patients survival was significantly better in patients with low AgNOR area per nucleus than in those with high AgNOR area, so AgNOR was reported to be an important prognostic factor (14). As a result of another study on MIB-1 positive cells in non-small cell lung cancer (15), the measurement of the AgNOR protein area was proposed in the evaluation of proliferative activity in tumor samples. In another study on patients with rectal cancer and evaluating the TAA/NA ratio, it was reported that the general and disease-free survival rates were significantly shorter in patients with higher TAA/NA mean (16).

There is no consensus on whether the AgNOR criteria used until now are useful in the differential diagnosis. Our AgNOR number findings were consistent with the studies of researchers (5, 13) who believe that the number and measurement of AgNOR in the literature may be unsuccessful or that there may be additional methods to aid diagnosis alone. Our mean TAA/NA ratio findings were also compatible with some of the studies (4, 12).

Various studies were performed about the use of AgNOR as a biomarker in xeroderma pigmentosum group E (17), testicular torsion (18), different doses of carbon monoxide poisoning in brain (19) and both heart (20) and lung tissue (21), ST-elevation myocardial infarction (22), clinical exacerbation of chronic obstructive pulmonary disease (23), colon adenocarcinoma (24), Ehrlich's ascitic carcinoma (25), comparison of fine needle aspiration biopsy and paraffin embedded tissue sections (26), renal ischemia/reperfusion (I/R) injury (27), hair root cells of humans (28 - 30), buccal epithelial cells of healthy individuals (31), developmental stages of Down syndrome infants (32), peripheral blood lymphocytes of babies/children with Down syndrome (33), oncocytology (34), etc.

Upon evaluation of the breast FNAB material, 10 were found to be suspicious, 6 nondiagnostic, and 4 false positive or negative. This means that the Pathology laboratory failed to perform correctly for 20 out of 46 patients.

Tables 1 and 2 show that the ratio of TAA/NA may be a good diagnostic criterion. When we examined the 20 patients for whom the Pathology laboratory did not give true results, TAA/NA ratios of the 4 patients who had benign results at the FNAB level but came out as having advanced malignancy were 3.77, 5.09, 6.38 and 7.9, respectively. Three of them were above the cut-off values of 4.83, which is compatible with the malignant outcome. The TAA/NA ratios of the 6 patients with nondiagnostic outcomes were 3.58, 3.64, 4.89, 8.98, 9.96 and 11.15, respectively. All these patients were diagnosed as malignant by advanced tests, and TAA/NA values of 4 of them were above 4.83. Of the 10 patients evaluated as suspicious, 4 were diagnosed as benign with advanced tests, and the TAA/NA values of these patients were 1.69, 2.92, 4.4, and 4.67, which were below the cut-off value, while the TAA/NA ratios of the malignant patients were 3.37, 4.01, 4.45, 5.78, 6.1 and 6.13, respectively. We thought that the results with deviations according to the cut-off value were due to the FNAB sampling made without fully entering the nodule.

As it is shown, the evaluation of the TAA/NA ratio in FNAB biopsy samples, specifically due to non-diagnostic aspiration, leads to more effective outcomes without requiring resampling. In this way, it would be possible to eliminate state expenditure, labor, patient hospitalization, wasted time spent by the health staff, psychological stress of the patient due to prolonged diagnostic treatment, through TAA/NA evaluation which is an inexpensive method.

To diagnose in breast biopsies, a minimum of 3 - 4 cell clusters should be existent and there should be 10 cells in each group, otherwise it is considered as nondiagnostic aspiration and requires additional biopsy material. In our procedure, there is no need to see the cells of groups and each cell is evaluated individually. Therefore, using FNAB without requiring the histopathological material, patients could be diagnosed. To gather more precise information on this matter, there is a need for additional studies including a large number of samples.

Our obtaining results using materials taken with a simple and easily applicable method, such as FNAB are compatible with more advanced but more invasive biopsy methods. Our results showed that it may be possible to diagnose the disease more safely by measuring the ratio of TAA/NA instead of evaluating the number of AgNOR. In addition, considering that breast has a distinct place for a woman among many other organs, we assume that patients

would have no aesthetic concerns if the material to be analyzed is taken by FNAB. It may be said that AgNOR parameters may provide important information about the prognosis of the disease and be useful to discriminate benign from malign lesion.

The morphology of the nucleus varies greatly depending on cell activity. The overgrowth of the nucleus is considered to be one of the most consistent cytologic features of cancer cells. In cancer cells, not only gene expression and its products but also cell morphology, size of cells and their nuclei were altered. For this reason, morphological changes in the nucleus are used to differentiate malignant cells from benign cells in tumor pathology (35).

The amount and distribution of AgNOR proteins associated with the nucleolus in malignant tissues can be used to understand the changes that occur in the nucleus in tumor pathology. As can be seen in Figure 2, while NORs in the breast cell nucleus of the control group and the benign group showed morphologically coherent and regular distribution, the NORs in the cell nucleus of malignant patient group were morphologically dispersed and irregularly distributed. In this way, benign and malignant cells can be distinguished from each other, but we think that a larger number of patients are needed to evaluate the patient groups at a level that will be able to distinguish them from their subgroups.

CONCLUSION

We have shown that TAA/NA ratio is more reliable than mean AgNOR number for the discrimination of malignant from benign breast lesions. We consider that our method provides a significant advantage over other methods in distinguishing benign lesions from malignant lesions in FNAB samples, since each cell can be evaluated individually without the need for cell groups.

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Komparativna evaluacija parametara argirofilnih regiona jedarnog organizatora kod benignih i malignih tumora dojke

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SAŽETAK

Cilj. Cilj rada bila je procena parametara argirofilnih regiona jedarnog organizatora (AgNOR) zbog razlikovanja benignih od malignih tumora dojke primenom novog pristupa – odnosa između ukupne AgNOR površine i nuklearne površine (TAA/NA).
Materijali i metode. Ispitanice uključene u ovu studiju podeljenje su u tri grupe: kontrolnu grupu (n = 14), grupu bolesnica sa benignim (n = 18) i grupu bolesnica sa malignim promenama (n = 28). Urađena je tehnika za bojenje AgNOR-a, urađena je procena srednjeg broja AgNOR-a i određen je odnos ukupne AgNOR površine i nuklearne površine (TAA/NA).
Rezultati. Dok su razlike između kontrolne i obe grupe bolesnica bile statistički značajne za broj AgNOR-a ($p < 0,001$), ova razlika između grupa bolesnica sa benignim i malignim promenama nije bila značajna ($p > 0,05$). Što se tiče odnosa TAA/NA, razlike između kontrolne grupe i grupe bolesnica sa benignim promenama ($p < 0,001$), između kontrolne i grupe bolesnica sa malignim promenama ($p < 0,001$), kao i između grupa bolesnica sa malignim i benignim promenama bile su značajne ($p < 0,05$).
Zaključak. Smatramo da procena TAA/NA stope, u poređenju sa brojem AgNOR-a može biti osetljiviji i korisniji alat prilikom razlikovanja benignih od malignih lezija dojke.

Ključne reči: **dojke, tumor dojke, regije nukleolarnog organizatora, AgNOR**