The Differences Proliferation Value of Argyrophilic Nucleolar Organizing Region (AgNOR) in Benign, Borderline, and Malignant Phyllodes Tumours of the Breast

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***Abstract*-** Phyllodes tumours is biphasic, because it is composed of neoplastic stromal cells and evolution containing epithelium. However, this stromal tumour is more cellular and increased. This tumours are far less common than fibroadenoma and de novo arising, and not from previous fibroadenoma tumours. The bad changes are the feared malignant are increased stromal cellularity, anaplasia, high mitotic activity, rapidly increasing tumour size, and infiltative edges. The incidence of phyllodes is <1% of all breast neoplasm in 0,1-0,5%, with the most incidence occurring at the age of 30 to 40 years.

Objective: This study was aimed to analyze the expression of AgNOR in benign, borderline, and malignant phyllodes tumours of the breast.

Methods: This is an observational analytic study with cross sectional approach, involving 35 paraffin block samples from phyllodes tumours. In this study we found mean AgNOR (mAgNOR) in 35 samples, consist of 15 samples of benign phyllodes tumours, 8 sample of borderline tumours, and 12 samples of malignant phyllodes tumours. The specimen of this study were embedded in paraffin and each section was cut form each block. Then AgNOR staining were done. The dots of AgNOR was counted in 100 nuclei and calculated to acquired mAgNOR count in each case. mAgNOR counts among 3 groups of cases were analyzed using statistic software.

Result: There were differences proliferation value of AgNOR between benign, borderline, and malignant phyllodes tumours.

Conclusion: The mAgNOR value malignant phyllodes tumours has the highest mean value, followed by borderline phyllodes tumours, and benign phyllodes tumours have the lowest mAgNOR values.

Index Term: AgNOR, Phyllodes Tumours.

I. Introduction

Phyllodes tumours is a rare fibroepithelial tumours of the breast. Phyllodes tumours account for 0,3-0,9% of all primary tumours of the breast.[1] Phyllodes tumours is the term for the biphasic neoplasm originally named cystosarcoma phyllodes by Johannes Muler in 1838, a term that is to be avoided because of its malignant conotations. Phyllodes tumours occurs in middle-aged and older woman. Very few patients are younger than 25 years age, which is in striking contrast with the age distribution of fibroadenoma. However, phyllodes tumours can certainly occur in young adults and even in adolescents, and therefore, the diagnosis cannot be excluded on the basis of age.[1-3] This tumours increased in Asian countries, in Singapore this tumours occurred 6,92% of all malignancies in the breast and occur at a younger ages, 25-30 years old. Although rarely found, there have been reports of phyllodes tumours in men. The frequency of these tumours events based on changes in histopathological features (gradations) is 75% benign, 16% borderline, and 9% malignant. Although it has been reported, there is rarely a synchronous or metachronous presence in this tumours.[4-7] Until now, it also difficult to diagnose phyllodes tumours because of subclassification resembling fibroadenomas. Because of the interest in cell proliferation, the value of Argyrophylic Nucleolar Organizing Region (AgNOR) has been evaluated in various lesions. Studies have shown significant differences in AgNOR scores for benign and malignant tumours of the breast. The usefulness of AgNOR scores lies in the assessment of tumour proliferation rates and in understanding cell kinetics and tumours.[8,9,11] AgNOR staining is a technique for detecting argyrophilia nucleolar regulatory proteins (NORs) associated. NOR is a ribosomal DNA loop responsible for transcription of ribosomal RNA on the short arm of the acrocentric human chromosome.[8] AgNOR is clearly seen in coloring as a blackish brown spot with 1000x magnification. This is the advantages of using network parts where clarity is only achieved at only 1000x magnification and the things of cutting influential network. AgNOR proliferation index is the percentages of cells that have a certain amount of AgNOR in each nucleus, this is considered a reflection of cells proliferation.[8,10,12] Therefore the mean AgNOR (mAgNOR) can be a better and more consistent indicator of changes in breast tumour proliferation.

II. MATERIAL AND METHODS

This is an observational analytic study, using a cross sectional approach. This study was conducted at the Department of Anatomical Pathology Faculty of Medicine Universitas Sumatera Utara, Department of Anatomical Pathology in H. Adam Malik Medan General Hospital, and Department of Anatomical Pathology in Pirngadi Medan Hospital. The research was held from November 2017 until May 2019, after approved by the Universitas Sumatera Utara and H. Adam Malik General Hospital Health Research Ethics Committee. All samples were obtained through surgical procedure. Inclusion criteria were phyllodes cases with adequate clinical data, available and undamaged formalin-fixed paraffin embedded tissue block with sufficient tumor tissue. Detailed clinical data were obtained from medical records or pathology archives consisting of age, sex, and size of the tumor. Histological type were determined independently by researchers through hematoxylin and eosin stained slides examination.

Histochemistry protocol and interpretation

The tissue sections were deparaffinized and rehydrated before pretreatment. AgNOR staining of section was done using routinely processed formalin-fixed, paraffin-embedded section from blocks in pathology files cut at 5-6µm. The basic staining procedures of ploton et al.[13] As used by Crocker was followed, with specific changes given in the result section. Section were deparaffinized in xylene. Section were hydrated through 100% and 95% ethanol or water. A silver-staining solution was prepared by dissolving 2% gelatin in 1% formic Acid at room temperature and filtering through filter paper. One part of the solution was mixed with two parts of 50% silver nitrate immediately before use. Staining was done at room temperature for 30-35 minutes. The sections were washed thoroughly in water , dehydrated in 95% and 100% ethanol, and mounted in a permanent mounting.[13] AgNOR expressions were determined independently by researchers. The expression in nucleoli was analyzed. Histochemistry staining of AgNOR was evaluated in terms of the proportion and staining intensity of tumor cells. The AgNOR was assessed on the count of mean AgNOR in 100 cells tumours nuclei (mAgNOR), with 1000x magnification, and we must use the emersion oil. The grading of dots dispersion was performed according to Khan et al.[14,15,16]

Statistical analysis

Statistical analysis was performed using SPSS software package version 22.0 (SPSS Inc., Chicago) with 95% confidence interval and Microsoft Excel 2010. Categorical variables were presented in frequency and percentage. Saphiro-Wilk test was applied to find out the normality continue data. The differences between benign, borderline, and malignant phyllodes tumours is assessed by one way ANOVA welch, and continue with post hoc Games-Howell test to assessed the differences between the groups. The p-values < 0.05 were considered significant.

III. RESULT

Patients' characteristics

The mean age for phyllodes tumour patients was 41.89 (±13.61) years. The most common in 12-63 years age group. Twenty-three patients (97.1%) were females, only 1 patients (2.9%) were males. The mean size of tumours was 21.85 cm which is 6-25 cm for benign phyllodes tumour, 12-15 cm for borderline phyllodes tumours, and 15-35 cm for malignant phyllodes tumours. The number of the patients benign phyllodes tumours was 15 (42.9%), borderline phyllodes tumours was 8 (22.9%), and malignant phyllodes tumours was 12 (34.2%). The histological subtypes of benign phyllodes was the majority of this case. Clinical basic characteristic of meningioma patients were summarized in table 1. Representative H&E sections are shown in figure 1.

Table 1. Characteristic of phyllodes patients

Age, mean ± SD, years	41.89 ± 13.61	
Benign 12-63		
Borderline 20-65		
Malignant 17-74		
Tumours size, mean, cm	21.85	
Benign 6-25		
Borderline 12-15		
Malignant 15-35		
Characteristic	N	Percentages(%)
Sex		
Female	34	97.1
Male	1	2.9
Diagnosis		
Benign phyllodes	15	42.9
Borderline phyllodes	8	22.9
Malignant phyllodes	12	34.2
TOTAL	35	100

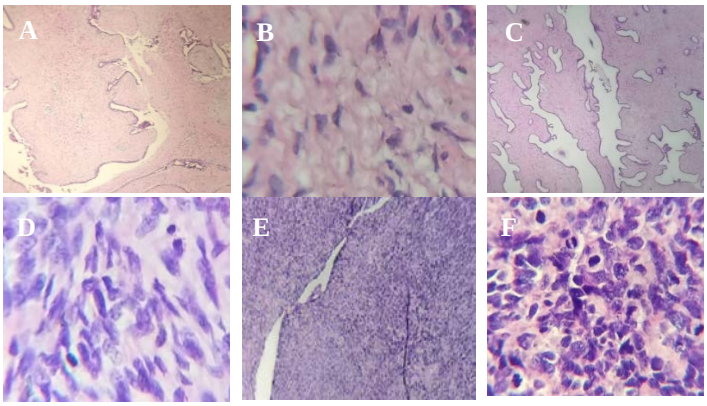


Figure 1. Histological type. A, Benign phyllodes (HE, 40x). B, Benign phyllodes (HE, 400x). C, Borderline phyllodes (HE, 40x). D, Borderline phyllodes (HE, 400x). E, Malignant phyllodes (HE, 40x). F, Malignant phyllodes (HE, 400x).

AgNOR expression

The intensity of AgNOR expression in nucleoli are shown in figure 2.

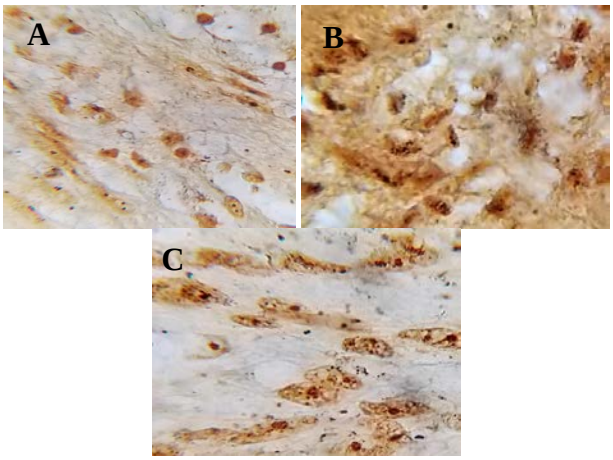
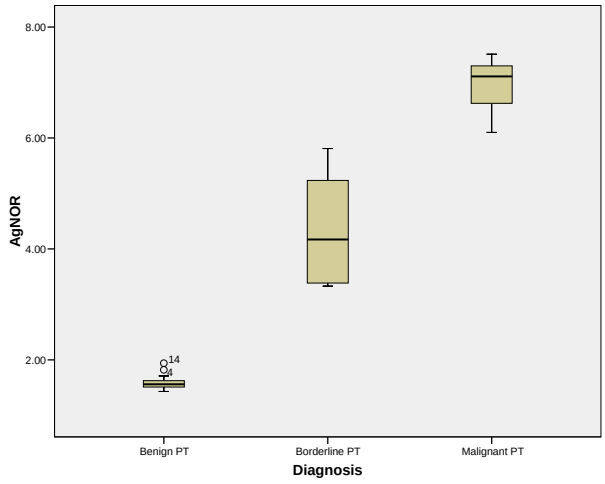


Figure 2. Histochemistry AgNOR expression. A, Benign phyllodes tumours. B, Borderline phyllodes tumours. C. Malignant phyllodes tumours.(AgNOR, 1000x)

Distribution of AgNOR's value in benign, borderline, and malignant phyllodes tumours

The evaluation of AgNOR histochemistry staining in phyllodes tumours can be either homogeneous and small, or regular or irregular and sometimes in the form of lumps. The AgNOR's dots are located in the nucleolus and colored blackish brown round to oval. The rest of the core is colored yellowish brown. In benign phyllodes, AgNOR dots appear smoother and fewer in number than borderline and malignant phyllodes tumours. Furthermore, the number of AgNOR dots can be determined at each cell nucleus in the stroma and carried out on 100 cells nuclei until the mAgNOR value is obtained. The mAgNOR value is calculated for each case and the group average value is obtained.

Tabel 2. Box Plot diagram of mAgNOR distribution value in each group of lesion.



In this study, we found that the distribution of the mAgNOR value in the lesion group is directly proportional, which means that the more malignant lesions in the phyllodes tumours, the higher the mAgNOR value.

Table 3. The comparison expressions of mAgNOR value of benign, borderline, and malignant phyllodes tumours

Diagnosed	N	mAgNOR Mean ± SD	p
Benign phyllodes	15	1.59 ± 0.14	<0,001
Borderline phyllodes	8	4.34 ± 1.01	
Malignant phyllodes	12	6.97 ± 0.44	
Total	35		

Table 4. The comparison mAgNOR value between group lesions of benign, borderline, and malignant phyllodes tumours

Phyllodes tumours	Mean diffrences	CI 95%		p
		Minimum	Maximum	
Malignant vs benign	5.37	5.03	5.72	<0.001
Malignant vs borderline	2.63	1.56	3.69	
Borderline vs benign	2.75	1.69	3.8	

There were differences in the mAgNOR values between groups of benign, borderline, and malignant phyllodes tumour lesions with p<0.001 and 95% confidence interval (CI). Clinically, there are differences in the mAgNOR values between malignant phyllodes tumours groups and benign phyllodes tumour with mean difference of 5.37. Between malignant and borderline groups there is a mean difference of 2.63. Whereas between the borderline and benign phyllodes tumour groups there was a mean difference of 2.75.

IV. DISCUSSION

The incidence of breast phyllodes tumours is very rare, with the most incidence occurring at the age of 30 to 40 years, even in one study reported phyllodes tumours occurred at an older age, ie 45-54 years. Tan et al. reported that phyllodes tumours were 6.92% of all malignancies in the breast and occurred at a younger age of 25-30 years.[1] In this study the mean age of the sufferers was 41.89 ± 13.61 years with the youngest age is 12 years, and the oldest age is 74 years. And most occur at the age of 12-63 years.

Karim et al., reported that although it was rarely found, phyllodes tumours had been reported report in men.[17] This was in line with this study where of the 35 samples studied, there was one male samples (2.9%), while 34 other samples were female (97.1%). The frequency of these tumour events based on changes in histopathological features is 75% benign, 16% borderline, and 9% malignant.[17] Tan et al., reported that the relative proportions

of benign phyllodes tumours were 60-70%, borderline 15-20%, and malignant at 10-20%.[1] Xiaofang et al., in their study reported from 52 patients there were 64% benign phyllodes tumours 25% borderline phyllodes tumours, and 6% malignant phyllodes tumours.[18] From this study we found 15 benign phyllodes tumours (42.9%), 8 borderline phyllodes tumours (22.9%), and 12 malignant phyllodes tuomors (34.3%).

The tumours can be clearly seen if it rapidly enlarges. Rapid enlargement does not always indicates malignancy. Looks shiny with stretched skin surface accompanied by widening of the skin surface veins. In cases that are nor handled properly, skin ulcers can occur due to tissue ischemia. Although proper skin changes in breast tumours always show signs of malignancy, but not phyllodes tumours, ulcer on the skin can occur in benign, borderline, or malignant lesions. Nipple retraction is not common. Ulceration indicates tissue necrosis due to large tumour suppression. Large tunours can also cause necrosis with bleeding.[19,20,21] Some previous studies found tumours to be less than 5 cm in size, therefore, the diagnosis cannot be made based solely on tumour size. Prolonged gaps (leaf-like appearance) in the across section are typical signs of phyllodes tumours.[22] In this study, the average tumour size was 21.85 cm, with a range of 6-35 cm. In benign phyllodes tumours the tumour size ranges from 6-25 cm, borderline 12-15 cm, and malignant phyllodes tumours 15-35 cm. Calhoun et al., in their study found that the phyllodes tumour size ranged from 1-40 cm. The results of this study indicate that most phyllodes tumours are large, however, there are also small-sized phyllodes tumours. In a study conducted by Flynn et al., and Calhoun et al., it was reported that phyllodes tumours were generally large (>2-3 cm).[20,21,23] This is suitable with the results of this study where it was found that phyllodes tumours size ranged from 6-35 cm.

The average value of calculation of AgNOR can be a way of assessing cell proliferation where the mAgNOR value is diagnostic in distinguishing between benign, borderline, and malignant lesions. This study used the WHO classification breast tumours involving 35 samples of phyllodes tumour consisting of 15 samples of benign phyllodes tumours, 8 samples of borderline phyllodes, and 12 samples for malignant phyllodes tumours.

From the 15 benign phyllodes tumour samples, the mAgNOR value was 1.59 ± 0.14. This value is lower than the results of the study reported by Machala et al. (3.2), and also lower than the AgNOR value obtained by study of Rajeevan et al. (2.7).[24]

In the group of borderline phyllodes tumour, the mAgNOR value was 4.34 ± 1.01. This value is not much different from the results of research conducted by Machala et al., which amounted to 3.18. While the group of malignant phyllodes tumours based on research conducted by Machala et al., Was equal to 4.98, and amounted to 5.37 ± 0.32 according to a study conducted by Rao et al., while in this study the mAgNOR value was 6.97 ± 0,44. This is also in line with the research obtained by Raymond et al.' (5.5 ± 2.3) and Chen et al. who found that >5 AgNORs in the cell nucleus.[25] This result are not statistically much different from those reported by Giri et al., with value of 4.4 ± 1.2.[26]

The study reported by Machala et al., found that AgNOR values in benign phyllodes tumour were 1.94, borderline phyllodes 3.18, and malignant phyllodes were 4.98.[27] Rao et al., in their study obtained an AgNOR value of benign phyllodes tumours of 3.2, borderline phyllodes 5.6, and malignant phyllodes had an AgNOR value of 5.37 ± 0.32.[28] This study shows that malignant phyllodes tumours have the highest mAgNOR values compared to benign and borderline phyllodes. This is suitable with research conducted by Machala et al., Rao et al., which states that the AgNOR value of malignant phyllodes tumour is higher than benign and borderline phyllodes tumours. Hena et al., and Iin et al., also reported that mAgNOR value in malignant lesions in the breast was higher than in benign lesions. Machala et al., also stated that the AgNOR value could be used to assess the proliferation of a tumour, and that AgNOR values were not much different in each group of tumours. This is suitable with this study which has a mAgNOR value that is not much different in each group.

The mAgNOR assessment of breast samples conducted by Machala et al., Rao et al., Hena et al., and Iin et al., showed significant difference in value between the mAgNOR values in benign breast lesions and malignant breast lesions. Rapid proliferation is part of the aggressive growth of cancer cells. In this study it was found that the mAgNOR value in benign phyllodes tumours was the lowest, followed by borderline phyllodes tumours, and the highest was malignant phyllodes tumours. Machala et al., reported that highest AgNOR value in malignant phyllodes tumours compared to benign and borderline phyllodes tumours, and AgNOR values can be used to assess proliferation of tumour lesions.[27] Mourad et al., reported the value of AgNOR is a reflection of the biological aggressiveness of breast cancer.[29] Egan et al., reported that the AgNOR value is an illustration of cancer cell proliferation activity. In another study it was reported that AgNOR values could be used to classify cancer tissue differentiation that cannot be done by HE staining from small biopsy and non patterned tissue certain.[30,31] Based on description above, the results of this study indicate that AgNOR histochemical staining can be used in the diagnosis of benign, borderline, and malignant phyllodes tumour of the breast, where AgNOR staining results are quite strong and reliable.

V. CONCLUSION

The average AgNOR value of benign phyllodes tumours is 1.59 ± 0.14, borderline phyllodes tumours 4.34 ± 1.01, and the mean values for malignant phyllodes tumours are 6.97 ± 0.44. There is a difference in the mean value of AgNOR between groups of malignant and benign phyllodes, between malignant and borderline phyllodes, and between groups of borderline and benign phyllodes tumours.

COMPETING INTERESTS

The authors have no relevant financial interest in the products or companies described in this article.

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ETHICAL APPROVAL

Health Research Ethical Committee, University of Sumatera Utara, Medan, Indonesia approved this study.

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