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Immunoexpression of human telomerase reverse transcriptase in fibroepithelial tumors of the breast

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ABSTRACT

Background: Phyllodes tumors and fibroadenomas are groups of fibroepithelial tumors in the breast that have different therapeutic approaches due to their rate of progression. The observed phenomenon can be attributed to the proclivity for recurrence and shared histologic characteristics, particularly within the fibroadenoma (FAs), benign phyllodes and borderline phyllodes. It's pathomechanism is undetermined yet. Human telomerase reverse transcriptase (hTERT) is one of the genes thought to play a role in the pathomechanism of this tumor.
Objective: This study aimed to determine the comparison of hTERT immunoexpression in the most common fibroepithelial tumor types.
Methods: This research was an analytic observational study with cross sectional method. The samples used were FAs and phyllodes tumors (PTs) which were divided according to the 2019 World Health Organization (WHO) classification. Histopathological examination was conducted on paraffin-embedded tissue blocks with FAs and PTs. Staining was performed using immunohistochemistry techniques with hTERT antibodies.
Results: A total of 69 samples were obtained consisting of 26 FAs, 17 benign PTs, 16 borderline PTs, and 10 malignant PTs. A significant difference was found in the loss of hTERT expression in fibroadenoma and phyllodes tumors in various grades. There was a positive correlation between hTERT immunoexpression and tumor type in this study (p= 0.0001).
Conclusion: There were significant changes in hTERT immunoexpression in fibroepithelial tumors, suggesting a role for hTERT in the pathomechanism of breast fibroepithelial tumors can be considered.

Latar Belakang: *Tumor fibroepitelial payudara merupakan salah satu kelompok tumor payudara yang mempunyai tatalaksana yang berbeda pada beberapa tipe tumor terutama pada fibroadenoma dan tumor filodes. Hal ini disebabkan kecenderungan rekurens dan, gambaran histologis yang mirip terutama pada kelompok jinak dan intermediet. Patomekanismenya masih belum diketahui hTERT merupakan salah satu gen yang diduga berperan dalam patomekanisme tumor ini.*
Tujuan: *Tujuan penelitian ini adalah untuk menganalisis imunoekspresi hTERT pada tipe tumor fibroepitelial terbanyak yaitu FA dan TF payudara pada berbagai macam gradasi.*
Metode: *Penelitian ini merupakan penelitian analitik observasional dengan menggunakan metode potong lintang. Sampel yang digunakan adalah tipe FA dan TF yang dibagi berdasarkan klasifikasi WHO 2019. Setelah dilakukan pemeriksaan histopatologi pada blok parafin FA dan TF, dilakukan pewarnaan dengan teknik imunohistokimia dengan antibodi hTERT.*
Hasil: *Didapatkan sampel sebanyak 69 terdiri dari 26 FA, 17 jinak, 16 TF borderline, dan 10 TF ganas. Didapatkan perbedaan yang signifikan dalam hilangnya ekspresi hTERT pada FA dan TF dalam berbagai gradasi. Analisis korelasi imunoekspresi hTERT dengan jenis tumor menunjukkan arah korelasi positif untuk TF (p=0.0001).*
Kesimpulan: *Hasil penelitian ini menunjukkan perubahan yang bermakna pada imunoekspresi hTERT pada tumor fibroepitelial sehingga diduga berperan dalam perkembangan tumor fibroepitelial payudara.*

INTRODUCTION

Histologically, FAs have several types most of which do not pose a diagnostic challenge, but cellular FAs and FAs types juvenile can have similar features with PTs and, admittedly, often have not clear assessments. Cellular FAs and fibroadenoma juvenile types are benign with biphasic morphology tumors, commonly known as different entities. Cellular fibroadenoma has an FAs architecture with gynecomastia cellular stroma and increased stromal cellularity.[1-3] Until now, it has been difficult to distinguish benign PTs from cellular FAs because increased cellular stroma is a prominent feature of both.[4,5] However, there are differences in therapy and prognosis in FAs and PTs.[6] Phyllodes tumors have several types of lesions with different clinical behavior, namely benign, borderline and malignant phyllode based on histopathological features that are in accordance with the classification of WHO recommendations.[1,7,8.] There are several recent findings that significantly explain the pathomechanism of fibroepithelial tumors, and support FA as a pure neoplasm. One of the genes thought to be involved in the pathogenesis of breast tumors is hTERT.[9,10] The role of the telomerase complex is thought to play an important role in cancer pathogenesis through this telomere-dependent or independent pathway. This mechanism includes complex changes in hTERT such as hTERT epigenetic changes, hTERT promoter germline, gene amplifications, hTERT structural variants, alternative lengthening of telomeres and somatic mutations. All of which were found to be specific at the tissue histotype level as well as at the single cell level. In these tumors, hTERT expression is regulated through genetic and epigenetic changes that ultimately influence telomerase activity. Telomerase activity through hTERT expression has an impact on telomere length so that this can be a useful marker in the diagnosis and prognosis of various types of cancer and the management of new therapies.[11] The possibility that FA rarely develops into malignant PTs has been shown by several studies. Recent analysis of breast fibroepithelial tumor types shows the presence of identical mutations in FAs and PTs (benign and malignant), thereby supporting research evidence that supports a clonal relationship between the FA and PT groups in the same patient. Pareja et al. found that in one patient with a history of breast tumors having three FAs, one PT and one malignant PT in the same breast, only the malignant PT, which was clonally related to one of the three FAs and had an hTERT promoter mutation. These findings support the theory that there is a possibility that FA changes are part of the development of PT, thus suggesting that genetic changes in hTERT will favor the development of malignant fibroepithelial tumors.[10] Although several studies show that this genetic factor is rare in FAs (0-7%), this mutation can play a role in the development of PT. The TERT expression is often found in the malignant PT group so it is thought that hTERT mutations may be useful in differentiating between benign PT and cellular FA.[12] This study aimed to determine the comparison of hTERT immunoexpression in the most common fibroepithelial tumors types, which can be very useful in establishing the diagnosis, pathomechanism and possible targets for further therapy.

METHODS

Study design

This study was an observational analysis with the cross-sectional method which was conducted in the Anatomical Department of Pathology Jambi Province Hospital in Jambi, a period of 2019 to 2020.

Population and sample

A total of 69 Formalin-fixed paraffin embedded (FPFE) that consisted of 26 FAs, 17 benign PTs, 16 PTs borderline, and 10 malignant PTs were obtained from the Anatomical Department of Pathology Jambi Province Hospital in Jambi, a period of 2019 to 2020. The FAs and PTs were divided based on the WHO 2019 classification.[2] Fibroadenoma is characterized by hyperplastic, myxoid and intracanalicular and pericanalicular features of the breast stroma and duct glands. Phyllodes tumors are characterized by stromal hypercellularity and the borderline PTs there are few glandular ductuli and are accompanied by atypical stromal cells in malignant phyllodes.

All cases were included in this study if they were diagnosed based on conventional histological parameters. The patients' age, tumor location, and relevant details were obtained from the pathology report. All cases were histologically classified based on stromal cytology atypia, stromal hypercellularity, number of mitosis, stromal

overgrowth, tumor necrosis, and tumor margins.

Hematoxylin and eosin (HE) staining

The paraffin block samples were cut using a Leica RM2135® microtome with a thickness of 5 µm and then attached to a slide. The tissue slides were then dried on a hot plate at 37-42°C for 3 hours. After that, the slides were immersed in absolute xylene twice for 5 minutes each, and then immersed in ethanol at concentrations of 100%, 90%, 80%, 70% and 50%, respectively for 3 minutes. After drying, the slides were stained with HE stains. The slides were then cleaned under running water, then again immersed in 90%, 95% 100%, 100% ethanol for 3 minutes each followed by two consecutive immersions in absolute xylene for 5 minutes each. Then the slides were covered with cover glass and kept under room temperature for at least 24 hours before observation under a light microscope for histopathological reassessment of tumor classification.

Immunohistochemistry staining

All samples were immunohistochemically stained at the Biomedical Laboratory, Faculty of Medicine and Health Sciences, Jambi University. In the initial step, a deparaffinization stage was carried out followed by rehydration of the sample for 30 minutes. Next, the rehydration stage was carried out with a gradual alcohol solution, namely 95%, 90%, 80% and 70%, soaking for 30 minutes in each group of alcohol. Then the sample slides were washed three times using distilled water and rinsed with phosphate buffer saline (PBS), followed by incubation in normal serum for 30 minutes and rinsed with PBS solution three times. Then incubated overnight with anti-TERT antibody (sc-377511, Santa Cruz, C-12) in the refrigerator, then rinsed again three times with PBS solution. The samples were then stained using 1,3-diaminobenzidine, then rinsed with distilled water and continued with the dehydration, clearing and mounting. Immunoexpression was scored as positive when intranuclear staining of tumor cells was detected. The number of positive cells was evaluated in 10 high power fields (40x) for each histological section and calculated as the percentage of positive tumor cells with a cut-off for positive staining is set at 20%; tumor cells with staining above this threshold are considered positive, while those below are deemed negative.

Data analysis

Data analysis using p-value is calculated based on the Chi-Square test on categorical data. If Chi-Square is not appropriate, then the alternative Kolmogorov Smirnov and Fisher's Exact tests are used. The significance value is based on p value <0.05.

Ethics

This research has been approved by the Ethics Committee of the Faculty of Medicine and Health Sciences, Jambi University (No. A/554/UN21.7/PT/2020).

RESULTS

After reassessment of all fibroepithelial tumor samples with a diagnosis of FAs and PT, 69 were obtained, consisting of 26 FAs, 17 benign PTs, 16 borderline PTs, and 10 malignant PTs (Table 1). The sample age range was 15 - 65 years with tumor sizes ranging from 1.3 cm to 21.7 cm, and were most commonly found in the left breast in 47 samples (68.1%).

Human telomerase reverse transcriptase immunoexpression were observed in stromal cell in one FAs (3.8%), 11 benign PTs (64.7%),

Table 1. Clinical characteristic of breast tumor

Variable	Percentage
Type	
Solitary	59(85.5%)
Multiple	10(14.5%)
Diagnosis	
Malignant Phyllodes	10(14.5%)
Borderline Phyllodes	16(23.2%)
Benign Phyllodes	17(24.6%)
Fibroadenoma	26(37.6%)

12 borderline PTs (75.0%) and all malignant PTs (100%). A significant difference in the loss of hTERT expression in PTs stromal was found compared to FAs (Table 2).

The correlation analysis of hTERT immunoexpression with tumor type showed a positive correlation direction for PT (able 3). For statistical analysis, the p-value is calculated based on the Chi-Square test, hTERT on epithelial and stromal cells obtained that p-value was less than 0.05, which means that there were differences between means for all variables that were statistically significant.

Figure 1 showed a comparison of hTERT

Table 2. Immunoexpression of hTERT in FAs and PT

hTERT	Fibroadenoma (n= 26)	Benign Phyllodes (n=17)	Borderline Phyllodes (n=16)	Malignant Phyllodes (n=10)	p
Epithelial					
Positive	14(53.8%)	14(82.4%)	14(87.5%)	6(60.0%)	0.065
Negative	12(46.2%)	3(17.6%)	2(12.5%)	4(40.0%)	
Stromal					
Positive	1(3.8%)	11(64.7%)	12(75.0%)	10(100.0%)	0.0001*
Negative	25(96.2%)	6(35.3%)	4(25.0%)	0(0%)	

hTERT: human Telomerase Reverse Transcriptase, FAs: Fibroadenoma, PT: Phyllodes Tumor
* p value<0.05 means for significant statistic

Table 3. Analysis of human Telomerase Reverse Transcriptase (hTERT) correlations with tumor type (FAs and PTs)

Variable	R	p-Value
Epithelial	0,543	0.065
Stromal	0.874	0.0001*

*p-Value < 0,05 means for statistically significant
FA: Fibroadenoma, PT: Phyllodes Tumor

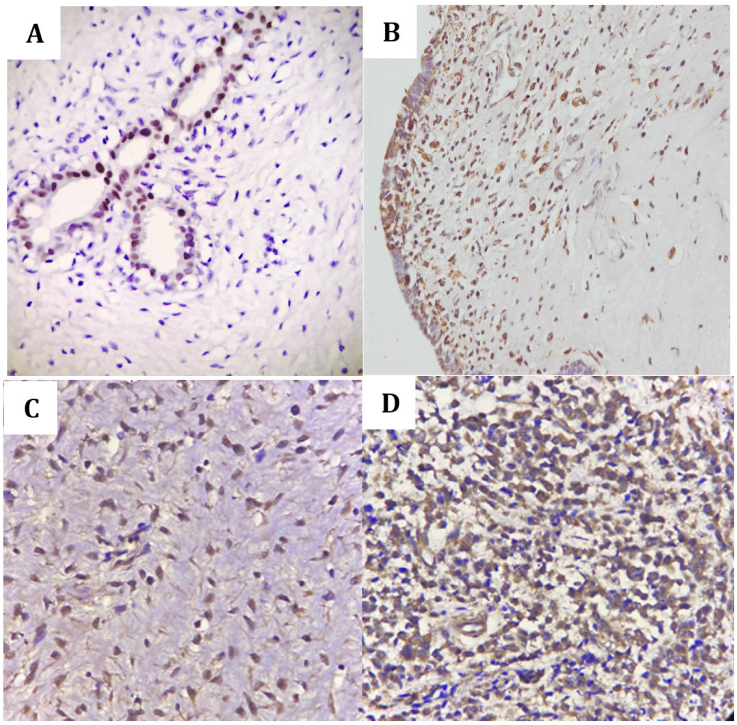

Figure 1. Immunoexpression of TERT (Olympus, 400x , IHC sc-377511,Santa Cruz, C-12)
a. Fibroadenoma b. Benign Phyllodes c. Borderline Phyllodes d. Malignant Phyllodes
TERT: Telomerase Reverse Transcriptase

immunoexpression in fibroepithelial tumors. It was shown that borderline (b) and malignant (c) PTs tumors show strong immunoexpression, and fibroadenomas show a weak distribution.

DISCUSSION

The latest WHO classification recognized that it is difficult to differentiate between fibroepithelial tumors correctly, especially between fibroadenomas and benign phyllodes tumors. The difficulty in accurately classifying fibroepithelial tumor lesions makes assessing the possibility of recurrence more difficult.[4,5] The current theory which proposes that telomeres in luminal cells shorten more rapidly compared to normal breast fibroblasts and myoepithelial cells can be considered the possibility that stromal protrusion may occur and contribute to the lack of telomere shortening in AF compared with ductal carcinoma in situ of the breast.[3–15] Further clarification is still needed through the use of in situ telomere detection methods and comparing infiltrating ductal carcinoma versus the breast epithelial carcinoma adenoma instead of fibroadenoma. [9,16] Not only do they have histological similarities, FA and PT also have the same genetic features. The presence of a clonal somatic mutation that targets exon 2 of MED12 and occurs repeatedly is found in FA and PT, so this is an early founder event. Importantly, among PTs, a gradual decrease in the prevalence of MED12 mutations from benign to borderline PT and to malignant PT was found.[17–19] Currently, MED12 mutations in PT are associated with hTERT promoter mutations, thus suggesting that these mutations may play a role in the synergistic pathomechanisms of this tumor.[13,20]

In this study, we found that the frequency of hTERT immunoexpression increased along with the histopathological grade of PT lesions, thereby supporting its potential role in the development of fibroepithelial tumors. This immunoexpression is mainly found in stromal cells but is rarely found in PT epithelial cells, so it is suspected that PT is a neoplasm in the mesenchymal group. The existence of hTERT strong expression in stromal cells in PTs malignant stromal cells is by the theory of pathomechanism of morphogenesis, which is suspected of changing epithelial cells into mesenchymal cells temporarily or permanently in fibroepithelial tumor cells, and referred to as epithelial-mesenchymal transition (EMT). In Poland in 2007 and Cold Spring Harbor Laboratories in 2008 which concluded three categories, one of which is related to neoplastic lesions (EMT type 3), which occur in neoplastic cells that previously undergo genetic and epigenetic changes in genes that support tumor cell development. These changes can be epithelial-mesenchymal transitions or occur due to mesenchymal-epithelial crosstalk.[4] The consistency of significant changes in hTERT immunoexpression in FAs and PTs grades in this study, can be considered helpful in differentiating the diagnosis of these tumors. Tsang found that overexpression of hTERT in stromal cells and the presence of promoter mutations were significantly associated with PT assessment and older patient age, and suggested implications for the pathogenic role of TERT alterations in the progression of PT.[21] Piscuoglio et al. found that mutations in the hTERT promoter hotspot were exclusively found in PT, and increased with increasing histopathological type; Consistent with this observation, the presence of hTERT promoter hotspot mutations was found only in the malignant PT group and not in FA.[22]

Several studies suggest that mutations in the hTERT promoter are thought to be responsible for hTERT overexpression in a variety of tumor types, including hepatoma, malignant melanoma and urothelial cell malignancies. Although there have been previous studies reporting telomerase activity in some phyllodes tumors and fibroadenomas, the mechanisms underlying telomere maintenance in these tumors have not been clearly understood.[16] Tan, et al found that mutations in cancer-related genes, such as retinoblastoma type 1 (RB1), epidermal growth factor receptor (EGFR), tumor protein p53 (TP53), and Neurofibroma type 1 (NF1), were only found in borderline and malignant PT groups, this is consistent with the theory that the PT mutation repertoire is thought to correlate with changes in histopathological grade.[18] Other genes, such as retinoic acid receptor alpha (RARA), filamen a (FLNA) and Set domain containing 2 (SETD2), are also mutated in both entities with lower frequency in FA, but until now it is not fully understood which gene is most responsible for the pathomechanism of fibroepithelial tumors. The existence of hTERT-stained epithelial cells in fibroadenoma in this study, it can be assumed that the process of changing these cells can trigger the continuation

of the formation of these cells to become more aggressive in the future. This is consistent with the presumed EMT mechanism. Further and specific research is needed to prove this theory. According to Kim et al., the TERT promoter region is the part where most genetic changes are found (70.6%), so TERT can be considered as a clue in therapeutic strategies in fibroepithelial tumors.[23] In this study, no analysis was carried out on the incidence of recurrence after surgery, because recurrence is one of the difficult problems in the management of fibroepithelial tumors, and also analysis incidence of FAs developing into PTs due to limited data.

CONCLUSION

There were significant changes in hTERT immunoexpression in fibroepithelial tumors, suggesting a role for hTERT in the pathomechanism of breast fibroepithelial tumors can be considered.

CONFLICT OF INTEREST

None declared.

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AUTHOR CONTRIBUTION

Fairuz (FZ): Conceptual framework, hypothesis, methodology and data analysis and preparation of articles. Humaryanto (HY): Sample collection, methodology, analysis of results, writing and editing.

LIST OF ABBREVIATION

hTERT: Human Telomerase Reverse Transcriptase; FA: Fibroadenoma; PT: Phyllodes Tumor, FFPE: Formalin-fixed paraffin embedded; PBS: Phosphate Buffer Saline; MED12: Mediator of RNA polymerase II transcription, subunit 12 homolog; TP53: Tumor Protein p53; RB1: Retinoblastoma type 1; FLNA: Filamen A; SETD2: Set domain containing 2; RA: Rheumatoid Arthritis; EGFR: Epidermal growth factor receptor; NF1: Neurofibroma type 1; EMT: Epithelial Mesenchymal Transition; RARA: Retinoic Acid Receptor Alpha, WHO: World Health Organization

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