Diagnostic test thyroid elastography examination and mRNA expression of the Cytokeratin-19 gene to determine the type of thyroid nodule

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ABSTRACT

Background: Thyroid nodules are still a health problem today. The incidence of thyroid nodules is still high. Thyroid nodules are found in 60% of the human population. Most thyroid nodules are benign, whereas malignant ones account for only about 10% of cases. Efforts are needed to determine the diagnostic value of ultrasound elastography and cytokeratin-19 biomarkers in determining the type of thyroid nodule.

Methods: This study is a diagnostic test on 42 thyroid nodule patients. Elastography examination using GE Logic 7 expert ultrasound and cytokeratin 19 biomarker examination using cubital vein blood.

Result: The study participant was dominated by 38 women and 4 men. The age range of the study subjects was dominated by the age range less than and up to 40 years with 27 people (64.3%) and at least in the age range more than 60 years with 3 people (7.1%). Elastography’s receiver operator characteristics (ROC) curve in determining thyroid nodules found that elastography’s ability to diagnose benign and malignant thyroid nodules was good with an area under curve (AUC) of 0.8409 (84.09%). ROC curve of cytokeratin-19 gene m-RNA expression in determining thyroid nodules found that the ability of cytokeratin-19 gene m-RNA expression in diagnosing benign and malignant thyroid nodules is excellent with an area under curve (AUC) of 0.9795 (97.95%).

Conclusion: Elastography has a good ability to determine the thyroid nodule while Cytokeratin-19 has an excellent ability to determine the type of thyroid nodule.

Keywords: Elastography; cytokeratin-19; Diagnostic test, thyroid nodule.


INTRODUCTION

To date the problem faced by clinicians and radiologists in managing thyroid nodules is determining an early diagnosis of benign or malignant thyroid nodules. This becomes important related to the subsequent treatment of nodules. A radiologist uses his modalities to determine whether a thyroid nodule is malignant or benign. The modalities used start from ultrasonography (USG), Computed Tomography Scan (CT Scan), Magnetic Resonance Imaging (MRI) to Positron Emission Tomography (PET).

In practice, ultrasound plays a significant role in determining the diagnosis of thyroid nodules, this is because ultrasound can determine the diagnosis of thyroid nodules up to 65%.

The use of ultrasound in determining thyroid nodules has both advantages and disadvantages. The advantages of using ultrasound include: non-invasive examination, relatively affordable, relatively available in almost all hospitals or other health care centers. While the weakness of using ultrasound is that it is operator-dependent, meaning that the results of the examination depend on the expertise or ability of the doctor who performs the ultrasound examination. Elastography as one of the features available on ultrasound can be used to evaluate thyroid nodules. However, the diagnostic value of elastography in determining thyroid nodules needs to be well recognized. In addition, it is also necessary to know the diagnostic value of cytokeratin-19 biomarker examination in determining the type of thyroid nodule.

Elastography is one of the features of ultrasound which is a non-invasive examination to evaluate the elasticity or stiffness (stiffness) of body tissues so that it can be used to evaluate benign and malignant thyroid nodules. Malignant nodules have a harder (less elastic) structure, so elastography can distinguish between benign and malignant nodules. Thyroid nodule elasticity can be evaluated using qualitative, semiquantitative, and quantitative methods. The choice of elastographic examination method depends on the needs and availability of...
the elastography features of the ultrasound device you have. A qualitative method evaluates the elasticity of nodules using color or color on the nodules combined with a 1-5 scoring system favored by the Tsukuba method. Furthermore, a semi-quantitative elastographic examination method called strain elastography. This method can be done in 2 ways, namely using the Elasticity Index (EI) and Elasticity Ratio (ER) values, hereinafter referred to as Strain Ratio (SR). The quantitative method of elastography is shear wave elastography (SWE) by giving a wave through a “push pulse” nodule and the speed of the wave propagating as it passes through the tissue will be detected as a basis for assessing the elasticity of the tissue. In this study, an elastographic strain examination was carried out using the Elasticity Index (EI) value. The elasticity index has a range of 0.0 to 6.0. The Elasticity Index (EI) shows the color distribution in the region of interest (ROI) relative to the ROI box. The higher the EI value indicates the less elasticity.

**METHOD**

**Research design and Participants**

This study is a diagnostic test study with a cross-sectional design on 42 research samples conducted at the West Nusa Tenggara Province General Hospital from November 2021 to June 2022 according to the inclusion and exclusion criteria. Inclusion criteria: patients with benign and malignant thyroid nodules and normal thyroid function (T4 = 6-12 mg/dL; T3 = 0.2-0.3 mg/dL), while exclusion criteria were patients not willing to participate in the study, patients with abnormalities (tumor) other than in the thyroid, the patient does not follow all examinations (elastography, examination of cytokeratin 19 gene mRNA expression and histopathological examination of postoperative tissue).

**Assessment elastography**

Elastography examination, the patient was examined in a supine position with a hyperextended neck using a GE logic 7 expert ultrasound machine with a high-frequency linear transducer (7-15 MHz) which provides adequate penetration and high-resolution images performed by a radiologist.

**Assessment of cytokeratin-19 expression**

**Nucleic acid extraction**

The sample volume is 100 µl of fresh vein blood added to 900 µl of “L6” solution consisting of 120 g of Guanidium thiocyanate (GuSCN) (Fluka Chemie AG, Buchs, Switzerland, cat no. 50990) in 100 ml of 0.1 M Tris HCl pH 6.4, 22 ml 0.2 M Ethylene Diamine Tetra Acetate (EDTA) pH 8.0 and 2.6 g Triton X-100 (Packard, Instruments) with a final concentration of 50 mM Tris HCl, 5 M GuSCN, 20 mM EDTA, 0.1 % Triton X-100. Then rotated at a speed of 12,000 rpm. The sediment was added to a 20 µl diatom suspension consisting of 50 ml H2O and 500 µl of 32 % (w/v) “Celite” (“diatoms”) (Jansen Chimica, Beerse, Belgium, 10.846.79). A total of 20 µl of this diatom suspension can bind 10 µg of blood RNA, then it is “vortexed” and centrifuged in a 1.5 ml Eppendorf tube at 12,000 rpm for 15 minutes. The supernatant was discarded and the sediment was washed with “L2” solution consisting of 120 g GuSCN in 100 ml 0.1 M Tris HCl, pH 6.4 by adding 1 ml of “L2” solution. Then it was vortexed and centrifuged at 12,000 rpm for 15 minutes, then the washing was repeated 2 times using “L2” solution, followed by washing with 1 ml 70% ethanol 2 times and 1 ml acetone. The result was then heated in a water bath at 56°C for 10 minutes and 60 µl of “TE” solution consisting of 1 mM EDTA in 10 mM Tris HCl pH 8.0 was added, then vortexed and continued centrifuged at 12,000 rpm for 30 seconds, then incubated in oven for 10 minutes at 56oC. Then vortex and centrifuge again for 30 seconds at 12,000 rpm and the supernatant is taken. The supernatant from this process will be obtained as a result of nucleotide extraction and stored at -80°C before PCR analysis.

**How real-time PCR works to determine the mRNA expression profile of the cytokeratin 19 gene**

The primary nucleotide sequence of human cytokeratin 19 (CK-19) mRNA used was CK-19 Forward: 5’-CTCACTACAgCCACACTACAGa-3’ (sense) and CK-19 Reverse: 5’-CTCAgCgCAgAgCCTgTT-3’ (antisense). Whereas the housekeeping used Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with the primary nucleotide sequence is GAPDH forward: 5’-TTgTATTgTggAAggACTCA-3’ (sense) and GAPDH reverse: 5’-TgTACATCATATTgCAggTTT-3’ (antisense). PCR conditions were 35 cycles consisting of 1 minute of denaturation at 95oC and annealing for 20 seconds at 54°C.

The amplification process uses specific primary oligonucleotides for surviving and cytokeratin 19 genes, where the housekeeping gene (internal control) is GAPDH, where qRT PCR uses the sybgreen qRT-PCR master mix kit, one stage. This protocol is optimized for real-time PCR machine CFX Connect System (USA) instruments. The protocol was adjusted using the instrument by changing the dye dilution based on the manual instructions and following the manufacturer’s recommended instrument for the RT-PCR cycle program.

Passive dye reference was included in the reaction, diluted 1:500. Solutions containing dyes are kept away from light. Dilute 2 x SYBR Green QRT-PCR master mix and store on ice. Following the initial defrosting of the master mix, the unused portions were stored at 4oC with notes, avoiding repeated freeze-thaw cycles.

The experimental reaction was prepared by adding the following components. Prepare the reagent mixture for the reaction using the following components. Reagent mixture by taking the final volume of 25 µl including the sample mRNA extracted according to the Hatta protocol, et al. 2017). A total of 12.5 µl of 2 x SYBR Green QRT-PCR master mix plus x µl of starting primer (optimized concentration) plus another Nuclease - PCR free - H2 level x µl of final primer (optimized concentration) and also 0.375 µl of reference dye solution from step 1 (optional) and 1.0 µl of the RT/Rnase enzyme block mixture to 25 µl of the total reaction volume can also be used. The reaction is mixed slowly so that bubbles do not form (not rotated), then distribute the mixture into test tubes by adding x µl RNA experiment to each
test tube. The reaction is mixed slowly so that no bubbles form (no rotation). The reaction was briefly centrifuged and the reaction was placed in the instrument and the PCR program ready to run using a Real-time PCR machine (CFX Connect system, Biorad Laboratories, Real-Time PCR 96 well 0.1 ml, USA).

**Histopathological examination**

A histopathological examination was performed by a pathologist using a paraffin block technique.

**Statistical Analysis**

Data analysis used SPSS 22 and Stata 16 software. For the proportion of sex, the Chi-square test was used, while the Mann-Whitney test was used to compare age proportions. Subsequent data analysis used the receiver operation characteristic (ROC) curve and the area under curve (AUC) using Stata 16 software. Data from ROC curve, a search for the optimal sensitivity and specificity cut of points was carried out for each elastographic variable and m expression. Cytokeratin-19 gene RNA on histopathology of benign and malignant thyroid nodules. After finding the optimal intersection point, then grouping thyroid nodules based on the intersection point is carried out. After that, it is processed using a 2x2 table. Then determined the diagnostic value of each variable as a marker of benign and malignant thyroid nodules.

**RESULT**

**Characteristics of study participant**

Characteristics of the subjects in this study (Table 1) obtained a total of 42 samples that met the inclusion and exclusion criteria. 38 women and 4 men dominated the research subjects. The age range of the study subjects was dominated by the age range less than and up to 40 years with 27 people (64.3%) and at least in the age range more than 60 years with 3 people (7.1%). Anatomic pathology examination showed that most of the dominant thyroid nodules had benign histology of 22 nodules (52.4%) and malignant histology of 20 nodules (47.6%). The histological type of benign thyroid nodules was dominated by Adenomatous Goiter 16 nodules (38.1%) and Well-differentiated neoplasm 3 nodules (7.1%) then Hashimoto thyroiditis 2 nodules (4.8%) and Solitary papillary hyperplastic 1 nodule (2.4%). Meanwhile, the histology of malignant thyroid nodules consisted of 20 nodules (47.6%). The location of the nodules was...
using Stata 16 software, the results of the diagnostic test for m-RNA expression of the cytokeratin-19 gene with anatomical pathology results with cutoff = 8589 fold change were obtained, Sensitivity 90%, Specificity 95.5%, Positive Predictive Value 94.7%, Negative Predictive Value 91.3%, Likelihood ratio (+) = 19.8, Likelihood ratio (-) = 0.105. A summary of diagnostic accuracy in elastography and cytokeratin-19 in conjugation to differentiate malignant and benign thyroid nodules can be seen in table 2.

**Table 2. Value of Area Under Curve (AUC), Cut off-point elastography and cytokeratin-19 in determining thyroid nodules.**

<table>
<thead>
<tr>
<th></th>
<th>Elastography</th>
<th>Cytokeratin-19</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC (%)</td>
<td>84.09</td>
<td>97.95</td>
</tr>
<tr>
<td>Cut of point</td>
<td>3.4</td>
<td>8589</td>
</tr>
<tr>
<td>Sensitivity (%)</td>
<td>75</td>
<td>90</td>
</tr>
<tr>
<td>Specificity (%)</td>
<td>81.8</td>
<td>95.5</td>
</tr>
<tr>
<td>PPV (95%CI)</td>
<td>78.9</td>
<td>94.7</td>
</tr>
<tr>
<td>NPV(95%CI)</td>
<td>78.3</td>
<td>91.3</td>
</tr>
<tr>
<td>Likelihood ratio (+)</td>
<td>4.13</td>
<td>19.8</td>
</tr>
<tr>
<td>Likelihood ratio (-)</td>
<td>0.306</td>
<td>0.105</td>
</tr>
<tr>
<td>Accuracy (%)</td>
<td>78.57</td>
<td>92.86</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The findings on the characteristics of the study subjects were dominated by female sex. This is consistent with some of the characteristics of a previous study by Waligorska et al. who conducted a study on the analysis of the DE3 variant survivin gene expression using 15 thyroid nodule tissue samples. The characteristics of the study subjects who were dominated by female sex were also similar to a study conducted by Yuen et al. who conducted a survivin IHC analysis study on 41 papillary thyroid carcinoma nodules.

Most thyroid nodules are benign, whereas malignant ones account for only about 10% of cases, besides that there are about 5% experience clinical symptoms due to nodule compression, and about 5% experience impaired thyroid function. Single thyroid nodule was found in half of patients with thyroid nodules. The prevalence of multiple nodules increases with age and in female patients.

A multicenter study conducted by Hairu et al. aimed to evaluate elastography’s diagnostic ability in 1445 thyroid nodules using the elastography score (ES) and Strain ratio (SR) values. ES has an AUC of 0.828 (82.8%) with a Sensitivity of 92.4%, a Specificity of 60.7%, a PPV = 78.9%, NPV 78.3%, Likelihood ratio (+) = 4.13 and Likelihood ratio (-) = 0.306.

m-RNA expression in ROC curve in figure 3 of the cytokeratin-19 gene in determining thyroid nodules, it was found that the ability of m-RNA expression of the cytokeratin-19 gene in diagnosing benign and malignant thyroid nodules was excellent with an area under curve (AUC) of 0.9795 (97.95%). This means that if m-RNA expression of the Cytokeratin-19 gene is used to predict malignant thyroid nodules in 100 people, then the right conclusions will be obtained in 97 people. From the results of analysis using...
There have been several previous studies evaluating the expression of the cytokeratin-19 gene in thyroid nodule tissue, including study by Erdogan et al. who analyzed cytokeratin (CK)-19 in thyroid nodules using the IHC method concluded that there were significant differences in CK-19 expression in groups of benign and malignant thyroid nodules; Kaliszewski et al. analyzed paraffin papillary thyroid carcinoma blocks of the classic subtype and concluded that CK-19 expression plays a role in the progression of papillary thyroid carcinoma (PTC) of the classic subtype.

In this study, examination of cytokeratin-19 gene expression using peripheral blood samples (cubital vein) concluded that examination of cytokeratin-19 gene mRNA expression in diagnosing benign and malignant thyroid nodules was included in the excellent category with an under curve area (AUC) of 0.9795 (97.95%).

Cytokeratin-19 (CK-19) is a biomarker found in both simple and stratified squamous epithelial tissue. Cytokeratin-19 is also found in expression in the gallbladder, hepatic duct, pancreatic duct, endometrium, fallopian tube, breast, bladder, lung, ovary, thyroid. However, CK-19 is unique compared to other cytokeratins in that it can be expressed in labile progenitor cells and is prone to transformation. CK-19 can function as a marker of premalignant transformation. In fetal skin CK-19 is found in the basal layer, whereas in adult human skin it is limited to the outer root sheath area of the hair follicle. This is a deficiency of CK-19 which is not specifically expressed in thyroid nodules.

CONCLUSION

Elastography has a good ability to determine thyroid nodules while Cytokeratin-19 has an excellent ability to determine the type of thyroid nodule.

CONFLICT OF INTEREST

All author declares there was no conflict of interest in research and publication.

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ETHICAL CONSIDERATIONS

This study received ethical approval from the Research Ethics Commission, Faculty of Medicine, Al-Azhar Islamic University (Number: 33/EC/-04/FK-06/UNIZAR/V/2022).

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Figure 3. ROC Curve of Cytokeratin-19 Gene m-RNA Expression in determining thyroid nodules.
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