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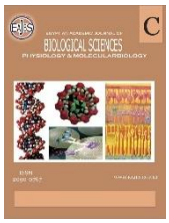

A Novel Non- Invasive Index for Thyroid Cancer Diagnosis Based on Nuclear Matrix Protein-1, Angiogenic Factor-1, and Hyaluronic Acid

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ABSTRACT

Background: The most typical endocrine cancer is thyroid cancer. Tumor biomarkers are genetic or protein-level compounds or specific evaluable traits. They assist in improving patient management. The goal of this study was to assess the ability of individual and combined potential markers (hyaluronic acid (HA), nuclear matrix protein (NMP-1), and angiopoietin-1) for thyroid cancer diagnosis. **Patients and methods:** This study registered 145 people (n=145), including 60 thyroid cancer, 65 benign group and 20 healthy controls. According to fine-needle aspiration (FNA) biopsy, Histopathological findings were divided into benign and thyroid cancer groups. The latter was further staged using the TNM approach, where (T) stands for tumor size and local invasion, (N) for nodal metastases, and (m) for distant metastases. We used biochemical routine profile tests like Total triiodoyhyronine (T3), Total thyroxine (T4), free Triiodothyronine (FT3), Free thyroxine (FT4), and thyroid-stimulating hormone (TSH). These measurements were done using Mini Vidas, Biomerieux, and ST-200 plus electrolyte analyzer (ELFA). We also measured HA, NMP-1 and ANG-1 by using Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) technique .**Results:** The diagnostic performances of single and combined markers were evaluated using area under Roc curve. An area under the Roc curve of 0.81, 0.62, and 0.67, respectively, was observed for hyaluronic acid, NMP, and ANG. The best marker for identifying thyroid is HA, A three-marker index called thyroid markers (hyaluronic acid, NMP, ANG) was developed. The thyroid mark's diagnostic performance at a selected cutoff index of 6.2. The thyroid marker's AUC was 0.87, its sensitivity was 86%, and its specificity was 80% when it came to distinguishing thyroid cancer from benign conditions. When distinguishing thyroid cancer from healthy individuals, the thyroid mark's AUC was 0.96, its sensitivity was 96%, and its specificity was 100%. **Conclusion:** HA, NMP-1 and ANG-1 are effective biomarkers to distinguish between thyroid cancer and benign with acceptable sensitivity and specifity.

ABBREVIATIONS

(ANG, Angiopoietin; ATC, Anaplastic Thyroid Cancer; AUC, Area under Roc curve; CK, Cytokeratin ; CRP, C-reactive protein ;DTC, Differential Thyroid Carcinoma; ELISA, Enzyme-Linked Immunosorbent Assay ; FNA, Fine Needle Aspiration ; FT3, Free Triiodothyronine; FT4, Free thyroxine ; FTC, Follicular Thyroid Carcinoma; HA, Hyaluronic Acid; IL, Interleukin; ; MTC, Medullary Thyroid Carcinoma ; NLR, Neutrophil to Lymphocyte Ratio ; NMP, Nuclear Matrix Protein; NPV: Negative Predictive Value ; OD: Optical Density; PLR: Platelets to Lymphocyte ratio.; PPV: Positive Predictive Value ; PTC: Papillary Thyroid Carcinoma; SD: Standard Deviation; Sens: Sensitivity ; SP: Specifity ; SPSS: Statistical Package for Social Science ; T3: Total Triiodothyronine; T4: Total Thyroxine; TIMP-1: Tissue Inhibitor Metalloproteinase ; TSH: Thyroid Stimulating Hormone; VEGF: Vascular Endothelial Growth Factor ; YKL-40: Chitinase-3-like)

INTRODUCTION

The most common cancer of the endocrine system, accounting for 3.4% of all cancers, is thyroid carcinoma (TC) (Prete A *et al.*, 2020). It makes up one-third of all endocrine cancers in Egypt and 1.5% of all malignancies overall. Nearly 3:1 of Egyptian females are impacted compared to males (Najadghaderi *et al.*, 2020). Radiation exposure, family history, and genetics are the key risk factors for thyroid cancer (Kruger *et al.*, 2022; Matrone, 2022). According to the World Health Organization's classification of histological TC types, follicular thyroid cancer (FTC) and papillary thyroid cancer (PTC), which together account for 80–85% of thyroid cancer cases, respectively (Lebbink *et al.*, 2022; Jukic *et al.*, 2022). Clinically, thyroid tumors (TC) can be divided into two groups: those with good differentiation (PTC and FTC) and those with poor differentiation (MTC and ATC) (Schlumberger and Leboulleux, 2021). Better detection technologies, such as the widespread use of thyroid ultrasound and an increase in the number of thyroid biopsies conducted, are the main contributors to the higher diagnostic rate for TC. However, the over diagnosis phenomena likely overweight the actual changes in TC epidemiology brought on by an increase in life expectancy, variation in iodine intake, and probable radiation exposure in isolated people (Matrone, 2021; Bogovic *et al.*, 2020). The main method for determining the early cancer risk of thyroid nodules and whether or not to conduct a fine needle aspiration biopsy is Sonography. FNA is invasive, hazardous, and affected by cytopathologist experiences (Wang *et al.*, 2019; Al-Ghanimi *et al.*, 2020). Sadly, patients with these unidentified tumors were only identified after undergoing thyroid surgery, and around 75% of them turned out to have benign neoplasms (Citgez *et al.*, 2013) Many studies have focused on blood markers, such as thyroid hormones, thyroglobulin, tissue inhibitor of metalloproteinase-1 (TIMP-1), calcitonin, chitinase-3-like (YKL-40), cytokeratin-19 (CK-19), and galactin-3 (Wang *et al.*, 2020; Agarwal *et al.*, 2021) for significantly improving the diagnosis of thyroid cancers and lowering the need for surgical intervention. This study was designed to evaluate the diagnostic performance of single and combined candidate markers (HA, NMP-1, and angiopoietin-1) to identify thyroid malignancies from benign tumors and healthy individuals.

MATERIALS AND METHODS

Patients:

A total of 145 Egyptians people including, cancer group (n=60), and benign group (n=65), in addition to healthy group (n=20), 125 (Benign and Cancer) with thyroid conditions underwent surgery. Thyroid surgery and postoperative pathological testing in the hospital were part of our selection criteria. According to fine-needle aspiration (FNA) biopsy, histopathological findings were divided into benign and thyroid cancer groups. The latter was further staged using the TNM approach, where (T) stands for tumor size and local invasion, (N) for

nodal metastases, and (m) for distant metastases (Casella *et al.*, 2018; Manzardo *et al.*, 2020). This study was approved by the Institutional Review Board and the Ethical Committee of the Faculty of Medicine at Mansoura University in Mansoura, Egypt, under the code number R.21.08.1415

Samples Collection and Preparation:

Prior to any surgical procedure, 10 ml of venous blood are drawn from each patient during the diagnosis phase. Two microcentrifuge tubes were used to aliquot the sera. The first aliquot was used for electrolyte profile and routine thyroid function test, which included measuring total triiodothyronine (T3), total thyroxine (T4), free triiodothyronine (FT3), free thyroxine (FT4), and thyroid-stimulating hormone (TSH). These measurements were done using Mini Vidas, Biomerieux, and ST-200 plus electrolyte analyzer (ELFA). Only before analysis did the second aliquot, which was kept frozen at -20©, for determined three candidate markers (HA, ANG and NMP-1).

Quantification of Nuclear Matrix Protein-1, Angiogenic Factor-1, And Hyaluronic Acid Using ELISA:

According to the manufacturer's instructions, HA, ANG-1, and NMP-1 (catalogue numbers E1374HU, E1222HU and E3052HU Changh, China, respectively) were each used in the Sandwich-ELISA technique to conduct the analysis using a microplate reader (Tecan Infinite F50 Austria GmbH, Austria) set to 450 nm, the concentration of candidate markers were determined by constructed a standard curve by plotting the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis and draw a best fit curve through the points on the graph. These calculations can be best performed with computer-based curve-fitting software and the best fit line can be determined by regression analysis. The optical density (OD value), which is proportional to the concentration of HA, ANG-1, and NMP.

Data Analysis:

A statistical analysis was performed using the Microsoft Windows version of SPSS 22.0. (SPSS Inc.). Quantitative/continuous variables were described as mean SD whereas categorical variables were described as frequency and percent distribution. The chi-square (X^2) test was used to compare categorical characteristics (such as thyroid cancer vs. benign outcomes) whereas the Student t-test was used to evaluate quantitative continuous variables. The Pearson correlation coefficient was used to assess the correlation. With a 95% confidence interval, the threshold for statistical significance was established at $P < 0.05$. Furthermore, the ideal cut-off values for the blood markers HA, ANG-1, and NMP-1 were identified by calculating the area under the ROC Curves (AUC). Regression models were created for the multivariate analysis utilizing various combinations of the diagnostic tools for each HA, ANG-1 and NMP-1

RESULTS

Patient's Characteristics:

A total of 145 patients, 20 healthy control (13.8 %), final histopathology revealed that 60 patients (41.4%) had malignant lesions which included Papillary carcinoma was the most common kind in 51 (35%) of the sixty malignant patients' diagnoses, followed by 8 follicular carcinomas (5.5%) and 1 medullary carcinoma (0.7%). , 65 patients (44.8%) had benign group, which included 27 colloidal nodular goiters (18.6%), 12 multiple nodular goiters (8.3%), 12 follicular adenomas (8.3%), 9 Hashimoto thyroiditis (6.2%), 3 thyroiditis (2.0%), and 2 hyperplastic nodules (1.4%).Table 1, shows Cancer group had elevated level of TSH than benign patients .and decreased level of calcium, ionized calcium and magnesium of benign than cancer patients.

Table 1: Levels of routine markers of studied groups**.**

Variable	Healthy	Benign	Cancer	P-value
Age (year)	45.6±11.9	41.7±12.2	44.2±15.0	0.6
Gender				
Female (no; %)	10 (6.9 %)	55 (37.9%)	38 (26.2%)	0.004
Male (no; %)	10 (6.9%)	10 (6.9%)	22 (15.2%)	
Thyroid function profile				
TSH (mIU/ml)	2.1± 0.5	6.5±1.1	7.2 ±2.6	0.148
T3 (ng/ml)	1.5 ±0.29	1.6±0.57	1.9±0.6	0.046
T4 (ug/dl)	7.4±1.0	8.5±3.88	8.4±2.4	0.46
Free T3 (pg/mL)	2.9±0.66	2.9±0.84	2.4±0.99	0.117
Free T4 (ng/mL)	1.24±0.7	2.17±0.5	2.36±0.15	0.445
Electrolyte				
Calcium (mg/dL)	9.0±0.15	8.6±0.85	8.0±1.18	0.009
Ionized Calcium (mmol/L)	1.08±0.13	0.88±0.31	0.77±0.22	0.001
Phosphorus (mg/dL)	5.36±0.57	3.7±0.57	4.0±0.28	<0.0001
Potassium (mmol/L)	4.2±0.46	4.4±0.88	3.4±0.97	0.019
Magnesium (mg/dL)	1.98±0.39	1.6±0.7	1.5±0.4	0.056
Sodium (mmol/dL)	140.9±3.1	137.0±1.86	137±9.3	0.169

Measurement of Candidate Markers:

Figure (1), shows that thyroid cancer had a higher level of HA (ng/ml) 186(65.4-432.6) than the benign 36.4(33.1-65.4) and healthy groups 35.4(34-55.6).In addition, the increase in NMP (ng/ml) for thyroid cancer was 82.2 (62.9-110.4), compared to 73.1(51-80.9) for benign conditions and 16.9(14.1-17.3) for healthy conditions also, Thyroid cancer had a higher level of ANG (pg/ml) 524.9 (399.6-765.6) than the benign group 432 (349-505.2) and healthy group 206.9(201-286.5). Table 2, showed the parameters of thyroid cancer patients' potential markers in relation to clinical pathological thyroid cancer.

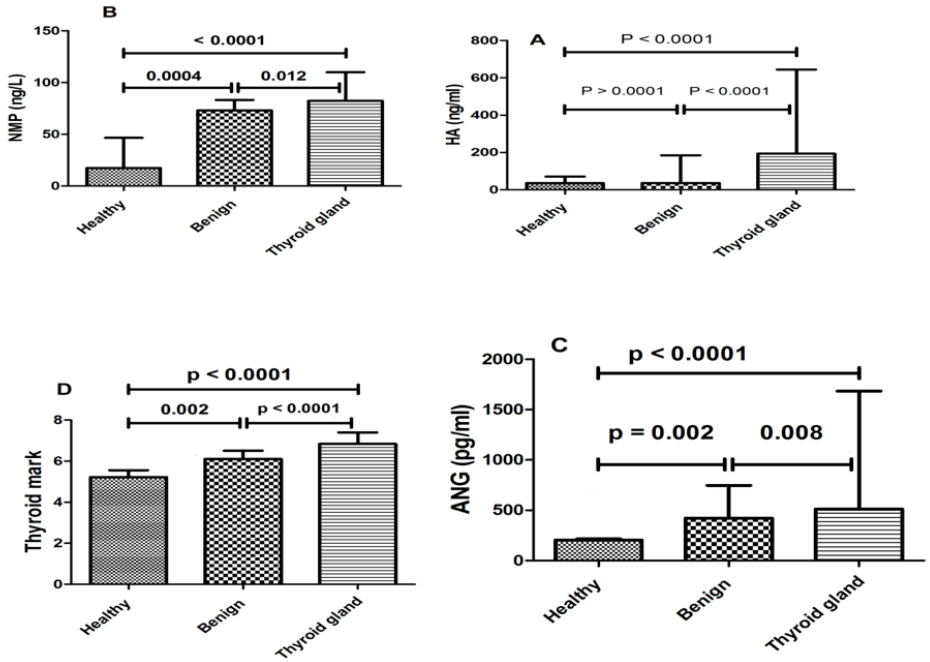

Fig. 1. Levels of candidate markers in studied groups. A. levels of HA. B. levels of NMP. C. levels of ANG. D. levels of thyroid mark

Table 2. Level of candidate markers with clinic-pathologic parameters of thyroid cancer patients.

	HA(ng/ml)	ANG (pg/ml)	NMP(ng/ml)	Thyroid mark
T stage, (no)				
T1 21 (32.8%)	221 (118-427)	490 (318 -797)	81(60.1-101.5)	5.6 ± 0.5
T2 25 (39%)	126 (43 -465)	538(425-684)	91.3(66.8-119)	8.6±0.67
T3 14 (21.9%)	174 (37-424)	505(402 -743)	76.6(47-98.9)	6.6 ±0.47
T4 4(6.25 %)	154 (92-291)	607(505-819)	78 (69-125)	6.8±0.48
P-value	0.001	0.016	0.06	< 0.0001
Tumor size(cm), no				
≤ 2 (23)	221(118-434)	499 (381-760)	81.5 (65.6-101.5)	6.8 ± 0.66
> 2 (41)	137 (43-429)	538 (404 -751)	83.0 (56.6-113)	6.9 ± 0.48
P-value	0.47	0.66	0.92	0.88
Lymph node status, no				
Positive (33)	166(100-432)	492 (394-783)	90.4(71.7-104.4)	5.9±0.51
Negative (31)	222 (41-431)	538 (426-751)	80.0(54.4-121.5)	6.8±0.53
P-value:	0.001	0.005	0.02	< 0.0001
Metastasis, no				
Positive (3)	428 (247-483)	607 ((496-623)	60.1(56-101.2)	6.7 ±0.57
Negative (61)	178(58-432)	512 (401-771)	83(64.2-104.4)	7.2 ±0.22
P-value:	0.001	0.005	0.02	< 0.0001

ROC Curves of Candidate Markers for Discriminated Thyroid Cancer from Healthy:

Hyaluronic acid, NMP, and ANG had respective cut-off values of 42, 50, and 369 that were most effective. Hyaluronic acid, NMP, and ANG each had an area under the curve of 0.84, 0.93, and 0.94; respectively figure 2. Among the possible markers, ANG is the most effective index for using healthy individuals to diagnose thyroid cancer. Table 3, showed the diagnostic abilities of potential indicators.

Table 3. Diagnostic performance of single and combined candidate markers

Marker	AUC(95% CI)	P-value	Cutoff	Sens	Sp	PPV	NPV	Efficacy
Thyroid cancer vs. healthy								
ANG	0.94 (0.74-0.94)	0.001	369	90	100	100	50	91
HA	0.84 (0.74-0.94)	0.013	42	80	80	97	28	80
NMP	0.93 (0.8-1.0)	0.002	50	86	80	97	35	85
Thyroid mark	0.96 (0.90-1.0)	<0.0001	6.0	96	100	100	71	96
Thyroid cancer vs. Benign								
ANG	0.67 (0.55-0.79)	0.015	468	60	100	100	20	64
HA	0.81(0.71-0.91)	<0.0001	42	80	80	98	29	80
NMP	0.62(0.48-0.75)	0.09	78	60	57	69	50	60
Thyroid mark	0.87 (0.79-0.95)	<0.0001	6.2	86	80	89	74	84

ROC Curves of Candidate Markers for Discriminated Thyroid Cancer from Benign Diseases:

Hyaluronic acid, NMP, and ANG had cut-off values with the greatest efficacy of 42, 78, and 468, respectively. Hyaluronic acid, NMP, and ANG each had an area under the curve of 0.81, 0.62, and 0.67, respectively. Among the potential markers, HA is the most effective index for detecting thyroid cancer in otherwise healthy people. Table 3 showed the diagnostic abilities of potential indicators.

Diagnostic Model:

To simplify, we used candidate markers without numerical constants and coefficients. We developed the thyroid mark, a special model that separates thyroid cancer patients from others with benign diseases. Thyroid mark= log (HA X NMP X ANG). Table 3 displays the simplified thyroid mark's

diagnostic performance at a chosen cutoff score of 6.2 (i.e., 6.2 showed absence of thyroid cancer, and >6.2 indicated presence of thyroid cancer). According to receiver operating characteristic curves, the thyroid mark's AUC was 0.87, its sensitivity was 86%, and its specificity was 80% when identifying thyroid cancer from benign conditions. When distinguishing thyroid cancer from healthy individuals, the thyroid mark's AUC was 0.96, its sensitivity was 96%, and its specificity was 100 %;(Fig. 2).

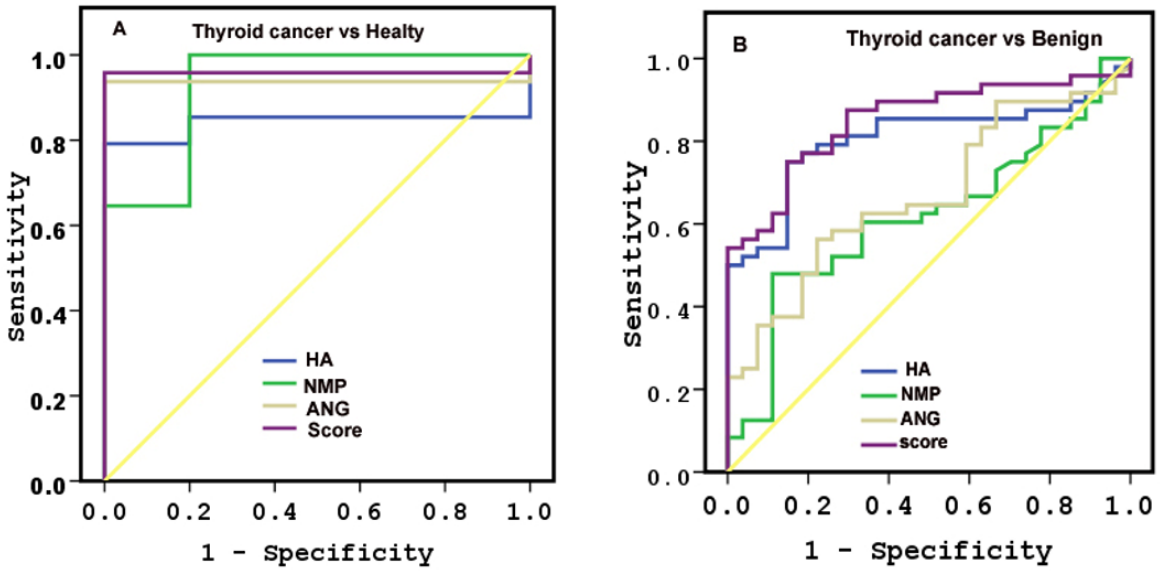

Fig. 2. Area under ROC curve of single and combined markers. A. Area under ROC curve of single and combined markers for discriminated thyroid cancer from healthy. **B**. Area under ROC curve of single and combined markers for discriminated thyroid cancer from benign diseases.

DISCUSSION

Although FNA is thought to be the primary approach for identifying thyroid cancer, it has a number of limitations, especially in separating follicular carcinoma from the more common follicular adenomas. An accurate diagnosis of thyroid cancer depends on the search for straightforward, non-invasive, sensitive, and useful indicators (Gomez Saez, 2011). The importance of HA, ANG, and NMP in predicting thyroid cancer has been greatly benefited. When glucuronic acid and N-acetyl glucosamine repetitions are connected by -1, 4 links, hyaluronic acid, a poly-anionic natural polymer, forms as linear polysaccharides. HA is a CD44 ligand, a protein that is frequently overexpressed in cancer cells and linked to the development of tumors (Safdar *et al.*, 2018). Thus, the levels of HA in this study were examined in malignant thyroid in comparison to healthy and benign thyroid. The findings showed that there was a highly significant difference in the level of HA between thyroid cancer patients and healthy and benign patients. Angiopoietin-1 is an oligomeric secreted glycoprotein that belongs to the angiopoietin family of growth factors. The capacity of Ang-1 to prevent endothelial cell death has received the most attention among its cellular effects. Angiopoietin-1 is overexpressed in human thyroid carcinoma relative to normal tissues, according to several studies (Kang *et al.*, 2017). The elevated expression of Ang-1 found in thyroid cancer patients in our study was extremely significant ($p < 0.0001$) and significant ($p=0.008$) when compared to healthy individuals. It was also highly significant ($p=0.002$) when compared to benign patients. The NMP has been shown to have a significant role in the development of neoplasia in recent years. It was established that the nuclear matrices of a number of experimental malignancies exhibited high-molecular weight phosphoproteins (Zbarsky, 1981; Kuzmina *et al.*, 1984). This study was

created to examine NMP-1 level in thyroid cancer patients using Sandwich ELISA in an effort to advance prior research; there is a notable increase in NMP-1 levels in the cancer patients. This research has focused on combining blood markers with straightforward investigative procedures for better efficiency of thyroid cancer diagnosis because no single thyroid cancer biomarker has sufficient sensitivity and specificity. As a result, the AUC value for detecting thyroid cancer increased from 0.62 for NMP; 0.67 for ANG -1 and 0.81 for HA; to 0.87 for thyroid mark. Additionally, the unique model sensitivity was noticeably higher than the individual sensitivities generated by each biomarker. In order to discriminate between benign and thyroid cancer, the combined markers raised sensitivity to 86%, specificity to 80%, and efficiency to 84%. Additionally, this thyroid model demonstrated a better AUC in earlier trials compared to other combinations. Using ELISA, Huang *et al.* (Huang *et al.,* 2014) demonstrated an AUC for the combination of VEGF-C, VEGFR-3, and TSH. Zhang et al (Zhang *et al.,* 2021) demonstrated that CRP and IL-27 were significant in relation to thyroid cancer (DTC). Additionally, the AUC for IL-27 between the papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC) groups was significant, and the AUC for NLR+PLR between the PTC and healthy control groups was significant as well. IL-27 and CRP were linked to DTC by multivariable logistic regression analysis. We acknowledge that limiting factor of failure to recruit a group with group consisting of different tumors other than thyroid cancer, and we will have done in the next research.

Conclusion

This is the first study to date to evaluate the diagnostic efficacy of index based on adding HA, NMP, and ANG to enhance the detection of thyroid cancer and possibly prevent unnecessary procedures. The development of a clinically useful test will eventually require research testing our model in sizable patient groups.

Declarations:

Ethical Approval: The study was approved by the Ethics Committee of Faculty of Medicine, Mansoura University, Mansoura, Egypt (Code # R.21.08.1415). Evaluation of Some Noninvasive Blood Markers for Diagnosis of Thyroid Gland Cancer. Informed written consent was signed by all patients in compliance with the ethical guidelines of the 1975 Helsinki

Conflict of interests: No potential conflict of interest.

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