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Expression of ARE-binding proteins AUF1 and HuR in follicular adenoma and carcinoma of thyroid gland

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Both adenylate-uridylate rich elements binding proteins AUF1 and HuR may participate in thyroid carcinoma progression. In this study we investigated the expression of both factors on a protein level with a special focus on follicular adenoma and follicular thyroid carcinoma. By employment of immunofluorescence and western blot on 68 thyroid tissues including 7 goiter, 16 follicular adenoma (4 adenomatous hyperplasia), 19 follicular thyroid carcinomas, 13 papillary thyroid carcinomas and 14 undifferentiated thyroid carcinomas we investigated protein expression of AUF1 and HuR. In addition to previous results we demonstrated that AUF1 and HuR are significantly up-regulated in carcinoma tissues as compared with follicular adenoma or goiter tissues. Furthermore, by evaluation of AUF1 or HuR expression, or combination of both proteins on total tissue lysates, we were able to demonstrate a significant difference between follicular adenoma and follicular thyroid carcinoma.

Overexpression of AUF1 and HuR is a common finding observed in thyroid malignancy. Analysis of the tissues obtained by surgical resection as demonstrated in this study is comparable to a fine needle aspiration and in combination with AUF1/HuR immuno-analysis may support the conventional immunohistological investigations. The promising results of this study were performed on relatively small collective, but justify future development of a quick thyroid diagnostic test on larger cohort of the patients, especially for thyroid samples which are inadequate for histological examinations.

Key words: AUF1, HuR, ARE, thyroid carcinoma, follicular adenoma, FTC

Thyroid cancer represents the most common endocrine malignancy [1]. Current molecular techniques employing genomics and proteomics revealed a rapid progress in identification and application of diagnostic markers for thyroid carcinoma [2]. However due to the different morphological interpretations, sample preparation and standardisation methods significant obstacles in utilisation of thyroid biomarkers are observed. These suggest a clear need to further search for new genes and/or proteins demonstrating diagnostic and/or prognostic potential. Still a growing body of evidence reveals that regulation of mRNA stability may affect thyroid specific functions and participate in processes of thyroid carcinogenesis. This regulation is largely exerted by trace elements such as iron, selenium and iodide, miRNAs and mRNA-binding proteins [3, 4, 5, 6, 7, 8]. Some of mRNA-binding proteins interact specifically with adenylate-uridylate rich elements (ARE) contained in 3' untranslated region (3'-UTR) of target mRNAs [9]. Out of many known ARE-binding proteins two of them, AUF1 and HuR, have been studied most extensively and demonstrated to regulate mRNA stability *in vivo*. Both proteins were reported to exert opposite effects on target transcripts. HuR is known to enhance their stability and/or translation, while AUF1 which is expressed as four isoforms causes generally the decay of some mRNAs [10, 11, 12, 13, 14, 15].

The patients with suspicious thyroid nodules are usually submitted to fine needle aspiration (FNA) biopsy as an initial test for histo(cyto)pathological evaluation. Papillary thyroid carcinoma (PTC) which is the most frequent diagnosed type of thyroid cancer is relatively easy to discriminate by employment of FNA biopsy. However in case of follicular thyroid carcinoma (FTC), follicular variant of papillary thyroid carcinoma (FVTC), and follicular thyroid adenoma (FA) definitive distinction of FA from FVTC, low-grade or well-differentiated FTC may be difficult or even impossible [16, 17, 18].

Current molecular approaches such as microarray analysis or proteomic studies were demonstrated to successfully dis-

criminate between FA and follicular neoplasms [19, 20, 21, 22, 23, 24, 25, 25]. However these methods are very expensive, time consuming and difficult to translate into clinical practice. Also immunochemical analysis and histo(cyto)pathological examinations are limited by tissue cellularity, shape and variable number of follicular cells, application of optimal antibodies and substrates or even needle size [27, 28, 29, 30, 31, 32].

Our previous studies demonstrated that ARE-binding protein AUF1 promotes thyroid carcinogenesis and may serve as a new additional marker for thyroid carcinoma progression [33]. Given that FNA biopsy samples may contain variable and limited number of thyroid epithelial cells and unwanted blood cells, we applied total tissue lysates obtained from surgical resection for investigation with specific AUF1 and HuR anti-sera. By employing western blot we tested the expression of these ARE-binding proteins in different thyroid specimens with a special focus on FA and FTC.

Patients and methods

Patients and tissue preparation. A total of 68 thyroid tissues, including 7 goiter tissues (G), 16 follicular adenoma

Table 1. Thyroid tissues employed in this study.

No.	Tissue	pTNM	Expression (%) as compared to positive control (C+)	
			AUF1	HuR
1.	Goiter		2.54	3.26
2.	Goiter		2.55	2.75
3.	Goiter		22.11	9.72
4.	Goiter		7.12	6.12
5.	Goiter		4.48	3.21
6.	Goiter		8.25	7.34
7.	Goiter		46.58	29.66
8.	Adenomatous Hyperplasia		145.77	47.31
9.	Adenoma		21.03	12.88
10.	Adenomatous Hyperplasia		38.31	30.35
11.	Adenoma		3.79	1.95
12.	Adenoma		4.08	4.86
13.	Adenoma		20.07	12.88
14.	Adenoma		1.62	3.38
15.	Adenoma		1.42	3.15
16.	Adenoma		7.87	5.03
17.	Adenoma		6.48	0
18.	Adenoma		12.87	0
19.	Adenoma		4.74	0
20.	Adenomatous Hyperplasia		70.19	49.29
21.	Adenoma		0	0
22.	Adenoma		0	0
23.	Adenomatous Hyperplasia		11.99	10.94
24.	FTC	pT4N0Mx	102.62	48.58
25.	FTC	pT4NxMx	52.7	55.60
26.	FTC	pT2N1M1	19.46	23.94
27.	FTC	pT3NxMx	23.27	40.02
28.	FTC	x	110.2	53.82
29.	FTC	pT4N1M0	69.24	78.13
30.	FTC	pT4N0Mx	29.42	21.55
31.	FTC	pT3NxM0	118.27	175.23
32.	FTC	pT3NxM0	30.16	26.72
33.	FTC	pT2N0Mx	14.61	15.34
34.	FTC	pT3N1Mx	47.57	40.92
35.	FTC	pT3N0M0	100.44	36.57
36.	FTC	pT3N0Mx	128.36	28.93
37.	FTC	pT2N1M0	120.6	56.78
38.	FTC	x	255.14	35.76
39.	FTC	x	237.46	138.04
40.	FTC	pT3N1M1	163.67	57.24
41.	FTC	pT4N0M0	67.82	53.98
42.	FTC	x	48.89	20.78
43.	PTC	pT2N0M0	93.83	38.83
44.	PTC	pT4N1M0	76.28	48.93
45.	PTC	pT1N0M0	82.64	144.24
46.	PTC	pT3NxM0	50.12	28.72
47.	PTC	pT4N1M0	85.97	58.59
48.	PTC	pT4NxMx	38.61	33.51
49.	PTC	pT4N1Mx	100.34	33.54
50.	PTC	pT4N1Mx	137.56	80.00
51.	PTC	pT4N1Mx	29.51	16.06
52.	PTC	pT4pN1Mx	101.67	36.38
53.	PTC	pT2N1Mx	90.70	38.61
54.	PTC	pT1N0M0	313.91	95.76
55.	PTC	pT4N1Mx	86.10	57.72
56.	UTC	pT3N1Mx	66.06	44.37
57.	UTC	pT3N1M1	106.85	82.80
58.	UTC	pT4NxMx	31.82	3.92
59.	UTC	pT3NxMx	121.15	77.37
60.	UTC	pT4	88.75	19.86
61.	UTC	pT4N0M1	112.39	70.18
62.	UTC	pT4N1Mx	104.72	104.66
63.	UTC	T4	60.95	36.56
64.	UTC	T4NxM1	80.67	93.15
65.	UTC	T4N1	49.89	46.94
66.	UTC	T4N0M1	114.96	72.51
67.	UTC	x	39.86	59.60
68.	UTC	T4	175.08	135.49

(FA) including 4 adenomatous hyperplasia tissues (AH), 19 follicular thyroid carcinomas (FTC), 13 papillary thyroid carcinomas (PTC) and 13 undifferentiated thyroid carcinomas (UTC), were collected directly from patients treated in the time interval from 1994 to 2011 in the Department of Surgery of the Martin-Luther-University Halle, by surgical resection. Pathological diagnosis of tissue sections was confirmed with haematoxylin and eosin staining. For 4 FTCs and 1 UTC sample the pTNM status was unknown (Table 1). All tissues were snap frozen in liquid nitrogen and stored at -80°C for further proceedings. For protein extraction the tissues with known diagnosis and histological classification were homogenized 2 times 20 sec at frequency of 2500rpm with Mikro-Dismembrator S (Braun Biotech, Melsungen, Germany). Homogenized tissues were incubated with extraction buffer containing 7M Urea, 2M Thiourea, 4% CHAPS and 40mM DTT. Lysis was performed at room temperature for 30 min, vortexing every 10 min. Supernatants were transferred into new tubes and the rest of un-dissolved tissue debris was discarded. 20µl of protein solution was applied for Bradford assay; the rest was stored at -80°C until use. In order to avoid the possible interference of lysis buffer with protein assay components, the samples were precipitated with chloroform/methanol method and then dissolved in 100 mM Tris prior to measurement. Total protein lysates obtained from FTC-133 cells representing follicular thyroid carcinoma, served as positive control and were prepared and handled the same way as tissue extracts. This study was approved by the ethical committee of the Martin Luther University, Faculty of Medicine, and all patients gave written consent.

Western blot analysis. Total protein lysates from tissues and FTC-133 cells (5µg) were resolved on 12% polyacrylamide gels and blotted onto PVDF membranes (Amersham Biosciences/ GE Healthcare, Uppsala, Sweden). Blocking was performed in 5% non-fat milk powder in 1xTBS /Tween 20 (0.1%) for 1h. After 3x washing with 1xTBS/Tween20, the membranes were incubated overnight with AUF1 (1:20000, Millipore, Schwalbach, Germany) and HuR H-280 (1:1000, Santa Cruz Biotechnology) anti-sera in blocking buffer. After washing steps, the secondary goat anti-rabbit sc-2004 (1:40000) antibody was used (Santa Cruz Biotechnology). Specific protein bands were visualised using the ECL Detection Kit (Amersham Biosciences). Densitometric evaluations of western blots were performed with Kodak Image Software 440 cf (Eastman Kodak). Expression of AUF1 was examined as total expression of all isoforms. The protein band representing positive control (C+) on each X-ray film analysed, was set as 100% and compared with other bands. Expression of AUF1 and HuR in all samples was normalised with b-actin. The protein expression is presented as % of C+. Detection of specific protein bands was repeated at least three times.

Immunofluorescence. Freshly cut cryo-embedded serial 6 µm sections of all thyroid tissues were fixed in cold acetone for 10 min. After 2 times washing with PBS/ Tween 20 (0.1%), the cells were incubated overnight at 4°C with the rabbit polyclonal antibodies against AUF1 (1:1000, Millipore, Schwalbach, Germany) or HuR H-280 (1:200, Santa Cruz Biotechnology) diluted with Dako Antibody Diluent (Dako, Glostrup, Denmark). Negative control sections were exposed to the secondary antibody only and processed as described below. After 3×10 min washing in PBS/ Tween 20 (0.1%), the cells were incubated for 1 h with a 1:200 dilution of TRITC-labelled anti-rabbit secondary antibody. After 1×10 min washing in PBS/ Tween 20 (0.1%), the slides were incubated in a 1:100 dilution in PBS-T of Hoechst staining (Hoechst AG) for 1 min. After 3x 10 min final washing steps in PBS/ Tween 20 (0.1%), the slides were dried and covered with fluorescence mounting medium (Dako). Finally, the cells were photographed under light/fluorescence microscope (Axioplan 2, Zeiss, Jena, Germany).

Statistics. Statistical analysis was carried out with GraphPad Prism software. Two-sided t test and multiple comparisons within thyroid tissues including Bonferroni correction were applied and p values *<0.01, **<0.001 and ***<0.0001 indicated statistical significance.

Results

Expression of AUF1 and HuR on immunofluorescent tissue sections. In order to assess the expression and localisation of both proteins, freshly cut thyroid tissues were stained with AUF1 and HuR anti-sera. As demonstrated in Fig. 1, both proteins were detected in all tissues investigated; however their expression varied between G, FA, and carcinoma tissues. G and FA demonstrated weaker and mostly nuclear expression of AUF1 and HuR as compared with strong, nucleo-cytoplasmic expression in carcinoma tissues.

Given that quantification of immunohistochemical slides is relatively difficult and depends on the subjective evaluation of the investigators, we focused further investigations on total thyroid tissue extracts by employment of western blots analysis. As FNA biopsies often contain variable and limited number of thyroid epithelial cells and unwanted blood cells, analysis of total protein obtained from surgical thyroid samples may be treated as FNA-comparable.

Expression of AUF1 and HuR on total tissue extracts. Protein lysates of thyroid tissues obtained from patients with known diagnosis were subjected for western blot analysis with specific AUF1 and HuR anti-sera (Fig. 2A). Similar to immunofluorescence studies, the expression of both proteins was noticeably weakly detected in G and FA in comparison with carcinoma tissues. Densitometric analysis of western blot results, revealed significant up-regulation of AUF1 and HuR in FTCs, PTCs and UTCs as compared with their low expression in FA and G (Table 1, Fig. 2B). The mean percentage expression for AUF1 in each histological subgroup was 13.38% (± 15.65, G), 21.89% (± 36.84, FA), 91.57% (± 70.49, FTC), 99.01% (± 72.78, PTC) and 83.77% (± 46.14, UTC). Analysis of HuR revealed following mean percentages: 8.87% (± 9.35, G), 11.38% (± 16.14, FA), 53.83% (± 42.01, FTC), 54.68% (±

36.26, PTC) and 60.53% (± 40.19, UTC). Furthermore and more importantly, the expression level of AUF1 or HuR, or combination of both proteins allowed significant discrimination between FA and FTC. Interestingly, in 3 cases of AH the expression of both proteins was noticeably higher than in other FA tissues investigated. The expression of AUF1 and HuR within thyroid carcinoma tissues was high, but did not significantly differ between FTC, PTC and UTC. We did not observe any correlations between AUF1 and HuR expression and age, gender and pTNM status.

Discussion

In this study we identified two ARE-binding proteins, AUF1 and HuR, as novel indicators of the malignant state of thyroid epithelial cell. We found that the low levels of both proteins in benign and adenoma tissues are extremely high in thyroid carcinoma tissues. Furthermore, in addition to our previously published data [33, 34], we were able to quantify the expression of both proteins on total protein lysates obtained from homogenized thyroid resectants. More importantly, by employment of AUF1 and/or HuR anti-sera on human samples often containing other and unwanted types of the cells (i.e. blood cells), we could discriminate between FA and FTC of the thyroid gland. To our knowledge, this is the first report demonstrating that quantification of those both proteins, especially AUF1, may possess the diagnostic properties for thyroid samples. With regard to HuR expression, its increased levels were demonstrated for malignant tissues of kidneys, skin, brain, cervix, tongue, gingival, colon, breast, ovary and stomach as compared to corresponding controls [35, 36, 37, 38, 39, 40, 41, 42, 43]. However most of these results were obtained by employing immunohistochemistry, which is often limited due to the technical reasons such as the quality and type of the specimens and antibodies used. These are especially important for thyroid samples where the quality of cell-block preparations is crucial for reliable immunostaining. Previous studies demonstrated that optimal staining with galectin-3, broadly reported as thyroid biomarker, requires formalin-fixed and paraffin-embedded preparations, biotin-free detection systems and application of specific monoclonal antibodies. Additionally, the conventional thyroid smears were reported to be inadequate for galectin-3 purposes [29], but its diagnostic properties are

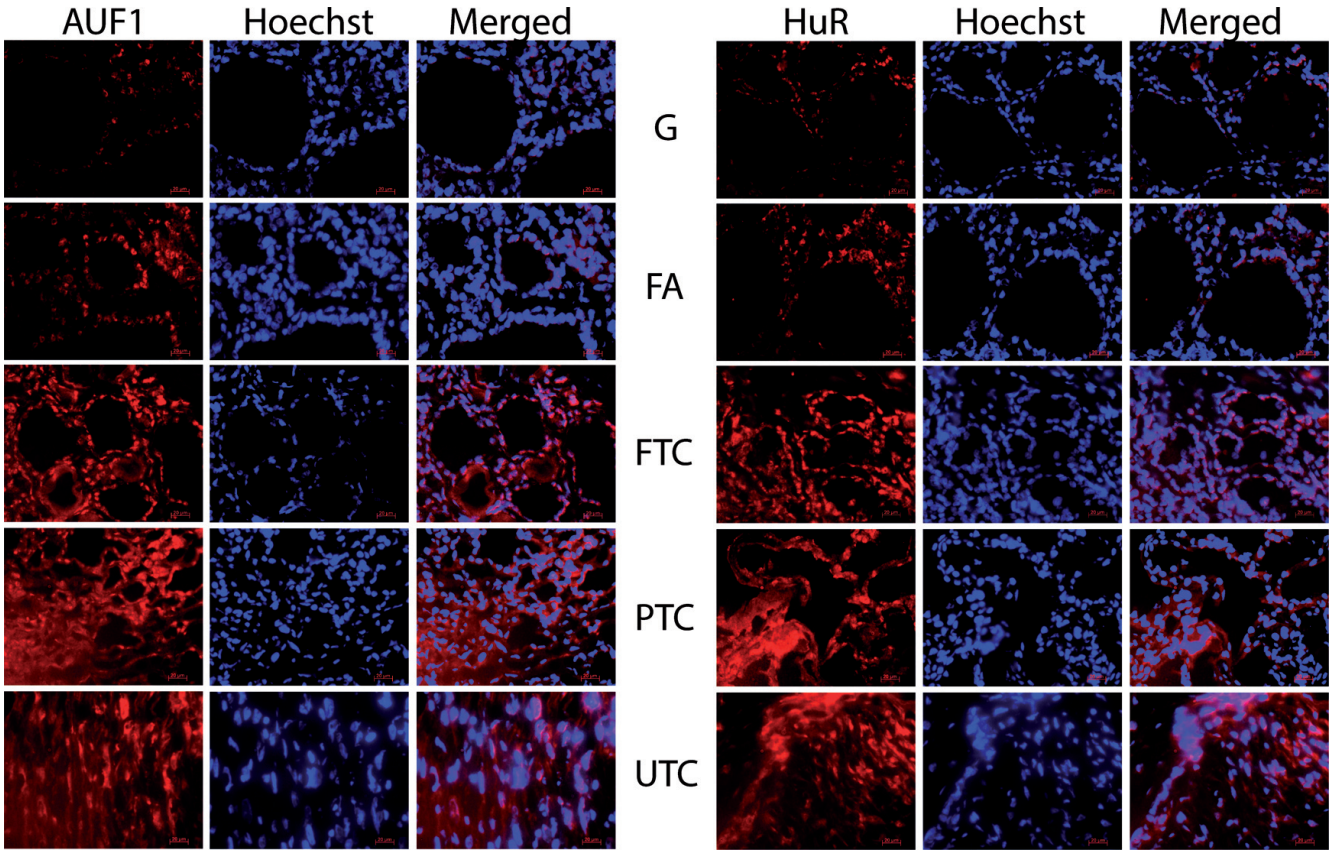

Figure 1. Immunofluorescence analysis of AUF1 and HuR expression on thyroid tissues. Frozen, freshly cut serial 6μm cryosections were stained with specific AUF1 and HuR anti-sera, and visualised with rhodamine-labelled secondary antibody (red). The nuclei were stained with Hoechst dye (blue). Note weaker and mostly nuclear expression of AUF1 and HuR in G and FA and stronger, nucleo-cytoplasmic staining in carcinoma tissues.

excellent on large-needle aspiration biopsy (LNAB) derived substrates [44]. Also the other markers including human mesothelial antigen (HBME-1), cytokeratin 19 (CK19), thyroid peroxidase (TPO) and keratan-sulphate (KS) or proteins related with energy metabolism such as alpha enolase (ENO1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), pyruvate kinase isoenzymes M1/M2 (PKM1/M2) and transketolase (TKT) employed to surgical thyroid or FNAB samples were reported to possess the diagnostic potential [45, 22]. Current investigations by Sponziello at al. revealed that employment of 4 proteins including peroxisomal proliferator-activated receptor-γ, serum deprivation response protein, osteoglycin and dipeptidase may support molecular discrimination between FTC and FA [46]. Also the employment of 5-gene classifier as demonstrated in study by Pfeifer at al. was successfully applied as a marker for FTC and FA in formalin-fixed paraffin-embedded samples [47]. Although the functional or biological rationale of the markers used in those studies remains not entirely understood for thyroid gland, they proved to be of a great accuracy.

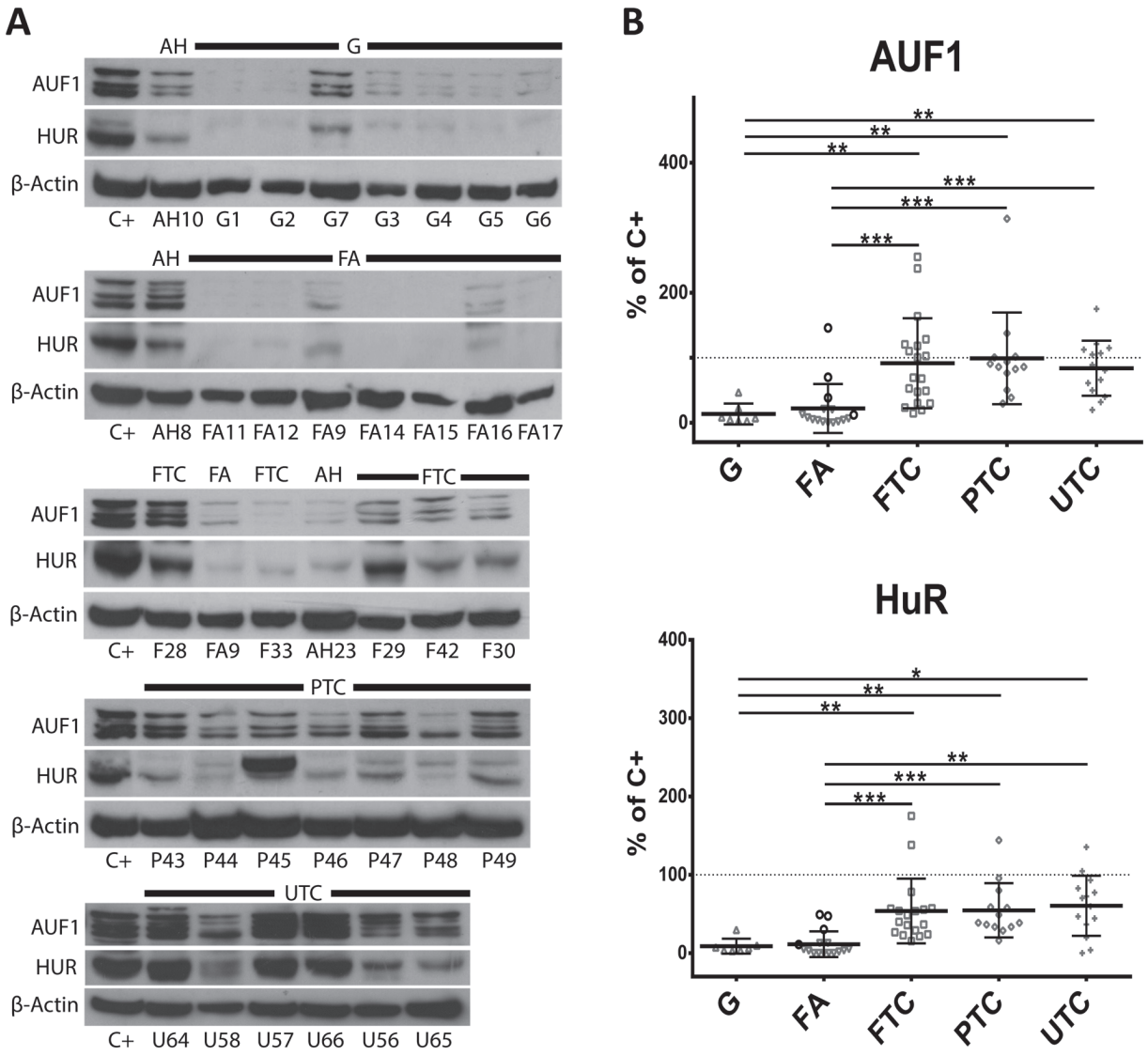

Figure 2. Western blot analysis performed on human thyroid samples. Total protein lysates obtained from goiter (G), follicular adenoma (FA), follicular thyroid carcinoma (FTC), papillary thyroid carcinoma (PTC), undifferentiated thyroid carcinoma (UTC) and FTC-133 cells were separated on polyacrylamide gels and blotted onto PVDF membranes. (A) Specific protein bands were detected with AUF1 and HuR anti-sera and visualised with ECL method on X-ray films. B-Actin served as normalising marker; C+, positive control; G1-G7, goiter; FA9, FA11, FA12, FA14-FA17, FA28, follicular adenoma; AH8, AH10, AH23, adenomatous hyperplasia; F28-F30, F33, F42, FTC; P43-P49, PTC; U56-U58, U64-U66, U69, UTC. The numbers after characters correspond to the numbers presented in Table 1. (B) Densitometric evaluation of western blot. The levels of AUF1 and HuR are expressed as % of positive control (C+) set as 100%; Note that AUF1 was evaluated as total expression of all isoforms; Means ± SD of % expression in all samples tested; Note that 4 AH samples in FA are indicated as bold circles; *<0.01, **<0.001 and *<0.0001 indicate statistical significance.**

In summary we report here that two ARE-binding proteins AUF1 and HuR are helpful to discriminate between benign and malignant thyroid lesions, but with this advantage that the quality of cytological preparation was omitted. This alternative immuno-analysis with both proteins performed on FNA-comparable surgical samples does not replace, but may support the conventional cytological investigations and future development of a quick thyroid diagnostic test, especially on thyroid smears for which definitive diagnosis is difficult.

The promising findings of this study were performed on relatively small collective but justify further investigations on larger cohort of the patients.

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