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ORIGINAL ARTICLE

E-cadherin/α-catenin deregulated co-expression in thyroid carcinoma based on tissue microarray digital image analysis

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Summary

Purpose: *Deregulation of cell-to-cell adhesion molecules is a common and also critical genetic event in epithelial malignancies leading to an increasing metastatic potential. Among them, e-cadherin and catenins – especially a and β – , act as oncogenes during the carcinogenetic process affecting specific signaling transduction pathways (i.e. Wnt/b-catenin). Concerning thyroid carcinoma, decreased or loss of expression in these proteins seems to affect the biological behavior of the neoplasm increasing its aggressiveness. The aim of this study was to investigate the deregulation of e-cadherin/a-catenin complex in thyroid carcinomas.*

Methods: *Thirty-five paraffin-embedded tissue samples including thyroid carcinomas (N=20) and also 15 cases of benign follicular nodules were cored at 1 mm diameter and transferred to a microarray block. Immunohistochemistry (IHC) was performed using anti- e-cadherin/a-catenin antibodies. Digital image analysis was also implemented for measuring the corresponding protein expression levels.*

Results: *E-cadherin/a-catenin protein expression demonstrated a significant progressive decrease regarding benign and malignant lesions (p=0.001). Simultaneous e-cadherin/a-catenin reduced or loss of expression was observed in 10/20 (50%) cancer cases correlated to advanced stage (especially nodal metastasis) of the examined tumours (p=0.02). Concerning the histological type, combined loss of e-cadherin/a-catenin expression was predominantly associated with follicular and anaplastic histology (p=0.001). Interestingly, a-catenin protein expression pattern was significantly correlated with the grade of differentiation of the examined malignancies (p=0.01).*

Conclusions: *Progressive loss of e-cadherin mainly and also a-catenin expression is associated with an aggressive phenotype (low differentiation, increased metastatic activity/advanced stage) in thyroid carcinomas. Based on their aberrant protein expression, novel agents have been developed for restoring their normal function.*

Key words: *cadherin, carcinoma, catenin, microarrays, thyroid*

Introduction

Thyroid carcinoma comprises a variety of pathological entities that arise mainly from follicular and parafollicular C-cells inside the thyroid gland. Papillary thyroid carcinoma and also follicular thyroid neoplasm/carcinoma represent the vast majority of them, whereas poorly differentiated anaplastic thyroid and medullary thyroid carcinoma are less frequent [1]. According to exten-

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sive epidemiological studies, thyroid carcinoma demonstrates an increasing incidence which has tripled over the past 30 years although its mortality seems to be stable [2,3]. Interestingly, in many cases thyroid cancer is characterized by a silent, asymptomatic subclinical profile, found at autopsy in people who died of other causes [4].

Among the genes that are involved in thyroid carcinoma genesis and progression due to genetic and epigenetic changes, cell-to-cell adhesion molecules deregulation drive tumor invasion increasing its metastatic activity [5]. Cadherins represent a super-family of calcium-dependent cell-to-cell adhesion glycoproteins comprised of an extracellular domain, a transmembrane region and a highly conserved cytoplasmic tail [6]. They mediate tissue differentiation, acting as suppressor factors. Recent studies indicate e-cadherin signaling is an important activator of c-Src at cell-cell contacts and is also the main regulator of actin-filament assembly through the e-cadherin/catenin complex regulated also positively by the 3,5,3'-triiodothyronine (T3). [7]. E-cadherin gene (CDH1) is located on chromosome 16 (16q22.1) and its functional loss – due to point somatic mutations or promoter methylation – in epithelial malignancies leads to a reduced or complete loss of its protein expression, inducing metastatic activity combined also with mutated β-catenin [8-10]. Similarly, catenins are a family of proteins found in complexes with cadherins promoting Ca2+ -dependent, homotypic cell-to-cell adhesion [11]. The first two catenins that were identified and cloned became known as α-catenin and β-catenin. Especially, α-catenin can bind to β-catenin and can also bind to actin. Concerning epithelial malignancies -including thyroid adenocarcinoma- e-cadherin/catenin complex disruption activates tumor metastasis mechanisms, increasing also the stage of disease [12,13].

In the current study we focused on the deregulation of e-cadherin/α-catenin complex in thyroid carcinomas based on tissue microarray protein analysis.

Methods

Study group

For the purposes of the study, 35 archival, formalin-fixed and paraffin-embedded tissue specimens in-

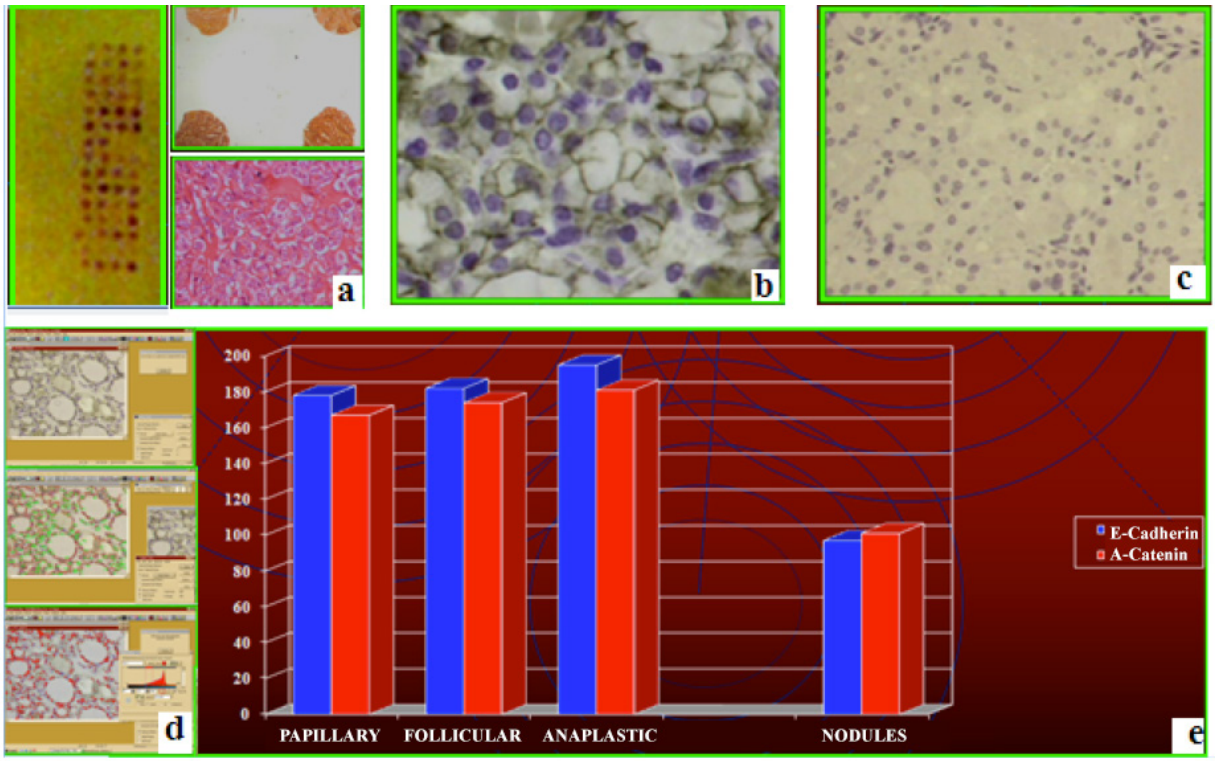

Figure 1. Combined e-cadherin/α-catenin protein expression analysis. **a:** Tissue microarray construction including all the examined thyroid cases (2 spots per case, H&E stain, original magnification 100x, 400x); **b:** Normal expression pattern for e-cadherin. Note continuous, round to nuclear brown, strong membranous staining (DAB chromogen, original magnification 400x); **c:** Loss of expression of α-catenin protein. Note complete absence of membranous/cytoplasmic staining pattern. Blue spots are referred to nuclei (DAB chromogen, original magnification 100x); **d:** Digital image analysis for evaluating e-cadherin/α-catenin protein expression. Green signals and reddish areas represent different protein levels (original magnification 100x); **e:** Histogram of combined e-cadherin (blue) /α-catenin (red) protein expression showing a significant difference regarding low to loss staining in thyroid carcinoma (increased digital values) and strong staining in normal-appearing follicular tissue spots (nodules) (p=0.001).

cluding 20 histologically confirmed primary thyroid carcinomas (15 papillary, 4 follicular and 1 anaplastic), and 15 cases of benign follicular nodules were used. All of the patients were female with average age 53.5 years. The initial diagnosis was performed by fine needle aspiration biopsy (FNAB). The hospital ethics committee consented to the use of these tissues in the Department of Pathology, 417VA Hospital (NIMTS), Athens, for research purposes, according to World Medical Association Declaration of Helsinki. The tissue samples were fixed in 10% neutral-buffered formalin. Hematoxylin and eosin (H&E)-stained slides of the corresponding samples were reviewed for confirmation of histopathological diagnoses. Similarly, FNAB samples were fixed by liquid-based cytology and stained using Papanicolaou (PAP) staining method. All lesions were classified according to the histological typing criteria of World Health Organization (WHO).

Tissue microarray construction

Areas of interest were identified in H&E-stained slides by a conventional microscope (Olympus BX-50, Melville, NY, USA). Selection of those areas was performed on the basis of tumor sufficiency, avoiding sites of necrosis or bleeding. Using ATA-100 apparatus (Chemicon International, Temecula, CA, USA), all of the source blocks were cored twice in order to secure the presence of each case in the final blocks and 1-mm diameter tissue cylindrical cores were transferred from each conventional donor block to the recipient block. After 3 μm microtome sectioning and H&E staining we observed microscopically that all of the examined cases were represented by at least one or two tissue spots (confirmation of the adequacy of the examined specimens) (Figure 1a).

Antibodies and immunohistochemistry assay

Ready-to-use anti-e-cadherin (clone 36b5, 1:40; Novocastra/Leica, UK) and also anti-α-catenin (clone 25b1, 1:50; Leica/Novocastra, UK) mouse monoclonal antibodies were applied in the corresponding tissue microarray spots. IHC for those antigens was carried out on 3 μm serial tissue microarray sections. The slides were deparaffinized and rehydrated. The NBA kit (Zymed-InVitrogen) was used. Blocking solution was applied to all slides for 10 min, followed by incubation for 1 h using the corresponding monoclonal antibodies at room temperature. Following incubation with the secondary antibody for 10 min, diaminobenzidine-tetrahydrocloride-DAB (0.03%) containing 0.1% hydrogen peroxide was applied as a chromogen and incubated for 5 min. Sections were counterstained, dehydrated and cover-slipped. For negative control slides, the primary antibodies were omitted. IHC protocol was performed by the use of an automated staining system (I 6000 Biogenex, CA, USA). Membranous predominantly and cytoplasmic staining was considered acceptable for the markers, according to manufacturers' data sheets (Figure 1b,c). Colon cancer tissue sections expressing the proteins and normal-appearing colon epithelia were used as control groups, respectively.

Computerized image analysis (CIA)

In order to evaluate the IHC results in an accurate and fast way, we performed CIA by using a semi-automated system with the following hardware features: Intel Pentium , MATROX II Card Frame Grabber, Digital Camera Microwave systems (800×600), Microscope Olympus BX-50, and the following software: Windows XP/Windows XP/NIS-Elements Software AR v3.0, Nikon Corp, Tokyo, Japan. Measurements for e-cadherin/α-catenin protein expression were performed in 5 optical fields per case and at a magnification of ×400 (Figure 1d,e). For objectivity reasons, we focused on representative tissue areas demonstrating even slight expression of the marker. Using normal non–neoplastic epithelia (benign follicular nodules) as control group, comparing them to the analyzed tumors, we characterized the corresponding expression levels as low to high as described in Table 1. A broad spectrum of 0-255 continuous staining intensity values (grey scale) was the basis for analyzing those different expression levels. Values increasing to 255 were correlated to progressively reduced protein expression, whereas decreasing

Table 1. Immunohistochemistry results and correlations

	*e-cadherin**			*a-catenin**		
	LL	*NE*	*p value*	*LL*	*NE*	*p value*
Histology			0.001			0.03
Thyroid carcinoma (n=20)						
Papillary (n=15)	15//15	0//15		6//15	9//15	
Follicular (n=4)	4//4	0//4		3//4	1//4	
Anaplastic (n=1)	1//1	0//1		1//1	0//1	
Control (n=15)						
(benign follicular nodules)	0//15	15//15		0//15	15//15	

* combined e-cadherin/α-catenin reduced/loss of expression: 10/20 vs nodal metastasis, p=0.02 and vs histology, p=0.001
LL: reduced or loss of expression (values 158-202), NE: normal expression (values 0-114)

to 0 values were associated with strong protein expression.

Statistics

Statistics were performed using the SPSS software (SPSS Inc., Ill, v. 11.0). Associations between variables including protein expression levels and pathological parameters were performed by applying the Spearman's correlation test. Two-tailed p values ≤0.05 were considered statistically significant. Total IHC results and correlations (p values) are described in Table 1.

Results

According to the digital image analysis decreased or loss of expression of e-cadherin was observed in all cases of the examined thyroid carcinomas compared to control group (benign follicular nodules), where a normal membranous protein level was detected (p=0.001). In detail, 11/15 papillary thyroid carcinomas demonstrated reduced protein expression and the rest complete loss of it. Additionally, in all of the follicular neoplasms reduced protein expression was observed, whereas the single case of anaplastic carcinoma showed complete loss of e-cadherin expression. Concerning α-catenin protein levels, different expression patterns were identified. In the majority of papillary carcinoma cases (9/15) normal or slightly decreased expression levels were detected. In contrast, follicular neoplasms showed a different intra-group expression (1/4 normal staining levels, 3/4 reduced expression), whereas the single anaplastic carcinoma case demonstrated significant decrease of protein's membranous expression. Simultaneous e-cadherin/α-catenin reduced or loss of expression was observed in 10/20 malignancies correlated to advanced stage (especially nodal metastasis) of the examined tumors (p=0.02). Concerning histological type, combined loss of e-cadherin/α-catenin expression was associated with follicular and anaplastic histology (p=0.001). Interestingly, α-catenin protein expression demonstrated a biphasic pattern in thyroid carcinoma cases correlated with the grade of differentiation of the examined malignancies (p=0.01). Low to moderate cytoplasmic expression with loss of membranous expression was detected in poorly differentiated papillary and anaplastic histology cases. E-cadherin was characterized by a monotonous membranous staining pattern in positive-stained cases.

Discussion

Extensive genetic analyses have already shown that a variety of altered molecular pathways are involved in the different histological types of thyroid carcinoma. MAPK, PI3K/AKT, IDH1-metabolic pathways are deregulated in all main cancer types including papillary, follicular and also anaplastic, excluding IDH1 in medullary histology [14,15]. Interestingly, WNT/β-catenin and p53-regulated pathways are deregulated especially in poorly differentiated and anaplastic carcinomas [16]. Although single activating somatic mutation of BRAF or RAS, and translocations producing RET/PTC oncogenes are significant genetic events involved in thyroid carcinogenesis, aberrant signal transduction of the WNT/β-catenin and also JAK/STAT3 pathways due to point mutations are correlated with an aggressive phenotype in these malignancies [17,18]. Some studies have shown that membranous β-catenin expression is progressively reduced with loss of tumor differentiation, resulting in tumor invasiveness, and increasing the corresponding metastatic potential [19]. Similarly, epigenetic modifications, which are referred to heritable changes in gene expression that occur without any alteration in the primary DNA sequence, are frequently detected in thyroid carcinoma. Aberrant methylation of CpG islands inside the promoters of specific genes that mediate crucial cell functions, including apoptosis, proliferation and differentiation or cell adhesion drive them to inactivation [20]. Concerning thyroid carcinoma, e-cadherin suffers its gene deregulation mechanism that leads to low or loss of its protein expression associated also to advanced stage, poorly differentiated or anaplastic histology [21]. Another mechanism of e-cadherin aberrant expression is the combination of allelic loss (16q) and point mutation in the remaining allele. This genetic event seems to be associated with an aggressive phenotype in papillary thyroid carcinomas due to suppressor genes, such as e-cadherin, inactivation [22]. Interestingly, another experimental study -based on a thyroid cell line analysis- showed that a point mutation in the exon 9 donor splice site caused a skipping of exon 9 with consequent deletion of the corresponding aminoacids on e-cadherin protein. This mutation led to a disturbed cell-to-cell adhesion although e-cadherin was still able to mediate the formation of the cadherin/ catenin complex [23].

Although the significance of e-cadherin/b-catenin in thyroid carcinoma biological behavior is well analyzed, the role of α-catenin expression is still under investigation. An experimental study based on embryonic mouse model analysis showed that conditional inactivation of the e-cad-

herin gene in thyroid follicular cells combined with α-catenin reduced on the cell plasma membrane affects gland development but does not impair junction formation. The authors concluded that e-cadherin has a role in the development of the thyroid gland and in the expression of β-catenin, but it is not essential for the maintenance of follicular cell adhesion [24]. This metabolic and functional event has been also analyzed on the basis of homotypic cell-in-cell structures in human tumor cells. Another basic research study discovered that the expression of α-catenin in these tumor cells restored cell-cell adhesion and promoted cell-in-cell formation in a ROCK kinase-dependent way. The study group identified α-catenin as another molecule in addition to e-/p-cadherin that were targeted to inactivate homotypic cell-in-cell formation in human tumor cells [25].

In the current study we co-analyzed the e-cadherin/α-catenin protein complex in thyroid carcinoma cases setting as control group cases of thyroiditis and benign follicular nodules. We observed that the majority of the examined tissue microarray cancerous tissues demonstrated reduced or loss of their protein expression. E-cadherin was correlated with a clear membranous staining pattern, whereas a cytoplasmic and membranous protein pattern characterized α-catenin localization. Interestingly, the cytoplasmic accumulation of the protein has been also observed by another study associated with an aggressive biological behavior of the neoplasm [26]. This event potentially explains the altered intracellular modification of the cytoskeleton protein complexes involving cadherins and the actin binding proteins vinculin and alpha-actinin during epithelial malignant progression. This genetic imbalance releases the metastatic process due to disruption of cell-to-cell adhesion, increasing also aberrant signalling transduction signals to the nucleus.

In our study, we also observed that simultaneous reduced expression of e-cadherin/α-catenin was correlated with advanced stage (especially nodal metastasis) in the examined thyroid carcinoma cases. Another study based on the expression and prognostic value analysis of α, β and γ catenins in differentiated thyroid carcinoma concluded that decreased α-catenin expression was associated with tumor recurrence, nodal metastases at the time of primary treatment and also follicular type of histology [27]. Additionally, we showed that progressive loss of e-cadherin mainly and also α-catenin expression are associated with an aggressive phenotype leading to anaplastic histology.

In conclusion, the role of e-cadherin/α-catenin aberrant expression in thyroid malignancies seems to be crucial for their metastatic progression. Based on the corresponding gene deregulation mechanisms, there is an increasing need for developing and evaluating inhibitory agents, such as CUDC-101 experimental protein which in vivo was associated with increased expression of p21 and e-cadherin, and reduced expression of survivin, XIAP, β-catenin, N-cadherin, and vimentin. This anticancer effect is very promising for targeting even anaplastic forms of thyroid carcinoma [28]. Besides those basic research results, understanding the role of GPX3 gene (5q23) silencing expression due to methylation in promoting tumor metastasis in human thyroid cancer should be a very useful molecular tool in identifying early-stage patients [29]. Finally, the involvement of CARP-1/CCAR1 (Cell division cycle and apoptosis regulator 1) in thyroid carcinoma, a peri-nuclear phosphoprotein, that plays a dynamic role in regulating cell growth and apoptosis by serving as a co-activator of β-catenin gene is under investigation [30].

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