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The Role of Epithelial Mesenchymal Transition Markers in Thyroid Carcinoma Progression

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Abstract

Understanding the molecular mechanisms involved in thyroid cancer progression may provide targets for more effective treatment of aggressive thyroid cancers. Epithelial-mesenchymal transition (EMT) is a major pathologic mechanism in tumor progression and is linked to the acquisition of stem-like properties of cancer cells. We examined expression of ZEB1 which activates EMT by binding to the E-box elements in the E-cadherin promoter, and expression of E-cadherin in normal and neoplastic thyroid tissues in a tissue microarray (TMA) which included 127 neoplasms and 10 normal thyroid specimens. Thyroid follicular adenomas (FA, n=32), follicular thyroid carcinomas (FTC, n=28), and papillary thyroid carcinomas (PTC, n=57) all expressed E-cadherin and were mostly negative for ZEB1 while most anaplastic thyroid carcinomas (ATC, n=10) were negative for E-cadherin, but positive for ZEB1. A validation set of 10 whole sections of ATCs showed 90% of cases positive for ZEB1 and all cases were negative for E-cadherin. Analysis of three cell lines (normal thyroid, NTHY-OR13-1; PTC, TPC-1 and ATC, THJ-21T) showed that the ATC cell line expressed the highest levels of ZEB1 while the normal thyroid cell line expressed the highest levels of E-Cadherin. Quantitative RT-PCR analyses showed that Smad7 mRNA was significantly higher in ATC than in any other group (p<0.05). These results indicate that ATCs show evidence of EMT including decreased expression of E-cadherin and increased expression of ZEB1 compared to well differentiated thyroid carcinomas and that increased expression of Smad7 may be associated with thyroid tumor progression.

Keywords

Thyroid carcinoma; Anaplastic thyroid carcinoma; ZEB1; Smad7; E-cadherin

Introduction

The aggressive behavior of anaplastic thyroid carcinoma (ATC) makes it one of the most lethal human malignancies with its rapid growth, invasion of adjacent tissues and metastases

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to distant organs (1, 2). Although recent studies have used multiple chemotherapeutic approaches to improve the prognosis of some patients with ATCs (2), it remains one of the most lethal cancers. Understanding the molecular mechanisms involved in thyroid cancer progression may provide targets for the effective treatment of ATC and other aggressive thyroid cancers.

Epithelial-mesenchymal transition (EMT) is a major pathologic mechanism in epithelial tumor progression. Local invasion and metastasis are linked to the acquisition of stem-like properties of cancer cells (3, 4). TGFbeta induces EMT by both Smad-dependent and-independent signaling events (5–11). TGFbeta1 ligand extends its signaling effects by activating a heteromeric receptor of two transmembrane serine/threonine kinases type I and type II receptors (TGFbetaR1 and TGFbetaR2). TGFbetaR2 transphosphorylates TGFbetaR1 by activating its kinase function and then phosphorylates intracellular Smad 2/3. The transphosphorylated Smads 2/3 associates with Smad4 and the activated complex is translocated to the nucleus where it interacts with other transcriptional co-activators and co-repressors to regulate expression of numerous genes (9–11). The Smad-dependent signaling which includes the stimulatory Smads (Smad 2/3, Smad 4 and inhibitory Smads including Smad7 regulates expression of various transcription factors that help to regulate EMT including Snail, Slug, Twist, ZEB1, and ZEB2 (9, 10). ZEB1/2 are important regulators of EMT during embryonic development and in cancer (11, 12). ZEB1/2 activates EMT by binding to E-box elements present in the E-cadherin promoter and suppressing its synthesis (13). ZEB1 also promotes EMT by repressing expression of basement membrane components and cell polarity proteins (14–17). Our recent studies showed that ATCs showed evidence of EMT with expression of high levels of Slug and Twist and low levels of E-cadherin compared to well differentiated thyroid carcinomas (18). To further investigate the role of ZEB1 and E-cadherin on EMT and on thyroid cancer progression, we examined the association between ZEB1, E-cadherin, and Smad 7 proteins in normal thyroid, benign thyroid tumors and in well differentiated thyroid carcinomas and ATCs.

Methods

Patient Material

Formalin fixed paraffin embedded (FFPE) tissues from 137 cases were used to construct a tissue microarray (TMA) as previously reported (18). Briefly, the TMA consisted of 10 normal thyroids, 32 follicular adenomas (FA), 28 follicular carcinomas (FTC), 57 papillary thyroid carcinomas (PTC) made up of 29 conventional PTC, 28 follicular variants of PTC (FVPTC), and 10 ATCs. The study was approved by the IRB at the University of Wisconsin Medical Center. The TMA consisted of triplicate 0.6mm cores made by using a manual tissue microarray (Beecher Instrument, Sun Prairie, WI, USA). The normal thyroid consisted of tissues from patients undergoing surgery for hyperparathyroidism or the opposite (histologically normal) thyroid lobe in patients with follicular or papillary carcinomas. Selection of regions in the tissue block for the cores included two cores from the periphery of the lesion and one core from the center of the lesion. A set of tissues consisting of 10 whole sections of ATC were used to validate the findings from the TMA.

Cell Lines

The normal thyroid cell line NTHY-ORI 3-1 was purchased from Sigma chemical (St Louis, MO, USA). TPC-1 cell line, which is a PTC cell line with RET-PTC rearrangement, but no BRAF mutation (19), was kindly provided by Dr. H. Chen (Department of Surgery, University of Wisconsin-Madison, WI USA). Both cell lines were maintained in RPMI and 10% FBS plus 1% penicillin/streptomycin at 37°C, 5% CO₂. The THJ-21T cell line was kindly provided by Dr. J. Copland (Department of Cancer Biology, Mayo Clinic,

Jacksonville, FL, USA) and maintained in RPMI with 10% FBS, 1% non-essential amino acids, 1% sodium pyruvate and 1% penicillin/streptomycin at 37°C, 5% CO₂.

Immunohistochemistry

For automated immunostaining, 5 microns thick tissue microarray sections were deparaffinized followed by heat-induced epitope retrieval using the Lab Vision PT module (Thermo Scientific) with Lab Vision citrate buffer pH 6.0. All immunolabeling was performed at room temperature using the Lab Vision 360 LV-1 Autostainer system (Thermo Scientific). All reagents used were from Biocare Medical, Chicago IL, USA, except where noted. Endogenous peroxidase was blocked for 5 min with Peroxidized 1. Nonspecific protein binding was blocked by Sniper and nonspecific avidin was blocked using the Avidin-Biotin kit, incubating 15 min for each reagent. Antibodies to E-cadherin (Cell Signaling Technology, Beverly, MA, USA, clone 24#10, 1:400), ZEB1 (Genway, 1/25), and Smad7 (Abnova, 1/500) were incubated for 60 min followed by incubation with biotinylated goat anti-mouse IgG for 15 min and subsequent 4plus Streptavidin-HRP treatment for 15 min. Beta-zoid Diaminobenzidine and Mayer's Hematoxylin were each incubated for 1 min. Primary antibodies were omitted in negative controls, which resulted in no staining. For positive controls, thyroid tumors previously shown to express the antigen of interest by immunohistochemistry and by RT-PCR were used. For manual immunostaining with the large tissue sections of ATC used in the validation studies, full tissue sections of the anaplastic thyroid carcinomas were deparaffinized followed by microwave heat-induced epitope retrieval with 10 mM citrate buffer pH 6 for 20 min. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 15 min followed by incubation for 30 min with the Universal Peroxidase Reagent Kit (Vector Laboratories, Burlingame, CA, USA) and developed with the ImmPACT DAB Kit (Vector Laboratories) and Gills Hematoxylin (Vector Laboratories) incubating each for 1 min.

The immunohistochemistry scoring was performed by two independent observers (CMG and RVL) using conventional bright field microscopy and differences in interpretation were reviewed for consensus. Unequivocal nuclear staining pattern for ZEB1, cytoplasmic staining for Smad7, and membranous staining for E-Cadherin were interpreted based on the intensity as negative, weak (1+), moderate (2+), and strong (3+). The expression was considered focal if positive cells comprised of 2–25% of all tumor cells in the tissue microarray sample and diffuse if this ratio was >25%. Focal staining of <1% of all tumor cells was considered to be negative.

RNA isolation and quantitative RT-PCR

Sections of formalin fixed paraffin embedded (FFPE) tumor samples (100 microns) were deparaffinized with succession of xylene and ethanol washes. Sections of cell lines were also used for RNA extraction. Total RNA was isolated with TRIzol Reagent (Invitrogen, Grand Island, NY, USA) according to manufacturer's instructions. RNA quality and concentration were assessed on the NanoDrop 1000 (Thermo Scientific, Pittsburgh PA, USA). Total RNA of 1 µg was reverse transcribed using the All-In-One First-Strand cDNA Synthesis Kit (GeneCopoeia, Rockville, MD, USA) with Oligo(dT) primer. PCR primers used were as follows: ECAD forward 5'-CCAGGAACCTCTGTGATGGA-3' and ECAD reverse 5'-TTTTGTGTCAGGGAGCTCAGGA-3', ZEB1 forward 5'-GCACAACCAAGTGCAGAAGA-3' and ZEB1 reverse 5'-GCCTGGTTCAGGAGAAGATG-3'', Smad7 forward 5'-TCACCTTAGCCGACTCTGC-3' and Smad7 reverse 5'-ACACCCACACACCATCCAC-3'. Annealing temperatures for mRNA primers was 58° C. The qRT-PCR was performed on the CFX96 PCR detection system (Bio-Rad, Hercules, CA, USA). Relative quantities were normalized to 18S and determined by the delta–delta CT method. Each experiment was repeated three times.

Statistical Analysis

Variables were assessed using χ^2 , or with Fisher's exact test and Student t test. Numerical variables were compared with one-way ANOVA. The statistical comparisons were performed using R software or GraphPad Prism version 5 software (San Diego, CA, USA) (18).

Results

Clinicopathological characteristics

The demographic and follow up information about the cases represented on the TMA are shown in Tables 1 and 2. Patients with ATC were older than patients in all other groups with a mean age of 74 years at diagnosis. The mean tumor size was significantly larger in the FTC and ATC groups ($p<0.001$, two tailed one-way ANOVA). Some of the FTC showed oncocytic features (48%). Four of the ATC had focal areas of PTC (two cases) or FTC (two cases) suggesting dedifferentiation from well differentiated carcinomas. The mean follow up ranged from 6 months to 46.5 months (Table 2). The most favorable clinical outcomes were observed in patients with PTC and the most aggressive tumors were in patients with ATC (Table 2). Five patients with ATC died of their disease while 2 patients died without disease and three patients were alive without disease. These 5 cases included patients who all had small foci of ATC which were discovered during surgery for PTCs. The 10 additional cases of ATC used as a validation set all had large cancers and they were all died from ATC within 14 months of diagnosis.

Immunohistochemistry

The TMA analysis is summarized in Table 3. ZEB1 staining was nuclear and it was expressed mainly by cases with ATC (Figure 1) with one patient with FTC also expressing ZEB1 (Table 3). The one case of FTC with ZEB1 expression was from a 58 year old man who had a 5.3 cm follicular carcinoma with capsular and vascular invasion. The patient subsequently developed lung metastases and died of thyroid cancer 5 years after surgery. The expression of ZEB1 was significantly higher in ATC compared to all other groups ($p=0.001$). ZEB1 immunostaining was also noted in some stromal fibroblasts and endothelial cells in the TMA (Figure 1E). E-cadherin membrane staining was expressed by all cases in the normal thyroid, in FA, FTC and in PTC (Figures 1A, B). Focal staining for E-cadherin was present in 3 of 10 cases of ATC, but was absent in all other cases. Smad7 was expressed in the nuclei of all thyroid tissues, but cytoplasmic Smad7 staining was lower in the normal thyroid compared to the adenomas and carcinomas (Figure C, F and I) (Table 3). The validation set of ten additional ATCs cases showed 9 cases positive for ZEB1 (90%) with diffuse staining in all cases; all cases were negative for E-cadherin.

qRT-PCR

Cell lines were used to analyze expression of ZEB1 mRNA since immunostaining of the paraffin sections showed that endothelial cells and stromal fibroblasts also expressed ZEB1 (Figure 2). The normal thyroid cell line had the highest levels of E-cadherin while ZEB1 was mostly highly expressed in the ATC cell line. Analysis of the FFPE tissues for Smad 7 mRNA expression showed significantly higher levels in the ATCs compared to the well differentiated carcinomas and normal thyroids ($p<0.05$).

Discussion

This study showed increased expression of ZEB1 in ATCs compared to well differentiated thyroid carcinoma and normal thyroid tissues. Our laboratory (18) and others (20–23) have

previously shown increased expression of Slug and Twist1 in anaplastic thyroid carcinomas compared to well differentiated carcinomas. Increased expression of ZEB1/2 and decreased expression of E-Cadherin has recently been reported in anaplastic thyroid carcinomas using molecular studies (16, 17). However this is the first demonstration of increased ZEB1 expression in ATCs using immunohistochemical analysis of paraffin embedded tissues to show nuclear localization. ZEB1 has been found to be associated with mesenchymal phenotypes in advanced pancreatic and other cancers in contrast to low expression in association with epithelial phenotypes (6). Studies of thyroid carcinomas have also shown that the leading front of PTCs has been associated with a more mesenchymal phenotype (24) suggesting EMT at the advancing front of thyroid carcinomas. Most studies of EMT in thyroid tissues have used ATC cell lines or tissues (20–23). However the study of Vasko et al with PTC tumors showed that the invasive front of the PTC overexpressed vimentin as well as TGFbeta, NFkappaB and integrin pathway members (24). They showed that EMT was common in PTC invasion and that vimentin regulated thyroid cancer EMT. The high expression of ZEB1 in primary thyroid carcinomas was significantly associated with repression of E-cadherin expression and with more aggressive carcinomas. This observation agrees with recent findings in pancreatic cancers in which ZEB1 and ZEB2 expression was associated with more aggressive behavior (6). Other studies have also shown that the miR200-family mediates posttranslational repression through sequence-specific binding to ZEB1 and ZEB2 mRNAs (16, 25, 26). The E-cadherin and N-cadherin molecules have different roles in EMT, since E-cadherin decreases during EMT while N-Cadherin increases (27). Curtis *et al* (27) showed that E-cadherin promote different phenotypes and behavior probably by interacting with proteins such as growth factors receptors and were responsible for the diverse cellular phenotypes observed when a cell expressed different cadherins (27). The molecular basis of EMT has not been completely elucidated (28). However the molecules involved in EMT may represent potential targets for pharmacological agents to control metastatic spread in the treatment of malignancies (28).

Quantitative qRT-PCR analysis showed significantly higher levels of Smad7 in ATC compared to the well differentiated carcinomas and normal thyroids. This suggests that high expression of this inhibitory Smad in ATC could contribute to the attenuation of canonical Smad's antiproliferative signaling in ATCs. Matsuo *et al* reported that in their immunohistochemical studies with normal thyroid and well differentiated thyroid carcinomas, there were higher levels of Smad7 in the carcinomas compared to normal thyroids (29). Other investigators have also noted a role of Smad 7 in other malignancies (30–34). Although Matsuo *et al* (29) reported mainly cytoplasmic staining for Smad7, Smad7 was present in both the nucleus and the cytoplasm in our studies. Eloy *et al* (35) analyzed Smad 2/Smad3, Smad4 and Smad7 in thyroid tumors and reported nuclear expression of Smad7 and noted that nuclear expression of this Smad protein was more frequent in the encapsulated lower grade PTCs. Smad7 inhibits apoptosis and growth arrest induced by TGF beta and activin (30, 31) and cytoplasmic Smad7 was noted to inhibit signal transduction of TGF beta as well as TGFbeta receptor activation (32). Upregulation of Smad7 has also been reported in other carcinomas including colorectal and endometrial carcinomas (34, 35), so this may be a common inhibitory mechanism in various cancers.

The use of E-Cadherin, ZEB 1 and Smad7 in recognizing more aggressive well differentiated thyroid carcinomas is premature at this stage and studies with the more aggressive variant of PTC such as tall cell carcinomas as well as widely invasive follicular carcinomas will have to be examined in future studies to establish the utility of these proteins as biomarkers for more aggressive thyroid carcinomas.

In conclusion, our study showed that ZEB1 is highly expressed in ATCs and is associated with loss of E-cadherin supporting a role for EMT in high grade thyroid carcinoma

development. The presence of increased levels of expression of Smad 7 in ATCs compared to well differentiated carcinomas suggests that increased expression of Smad7 may also be associated with tumor progression in thyroid carcinomas.

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Figure 1.

Examples of E-cadherin, ZEB1 and Smad 7 immunostaining in TMA sections of formalin-fixed paraffin-embedded sections of thyroid tissues (A–I). Membranous staining for E-cadherin was present in the normal thyroid and PTC (A and D) while most ATC were negative for E-cadherin (H). ZEB1 was expressed in the nucleus of most ATCs (IH), while the normal thyroid (B) and PTC (E) showed ZEB1 staining in the stromal fibroblast, but not in the neoplastic follicular epithelial cells. Smad7 showed nuclear staining in all cases; cytoplasmic staining and was higher in the carcinomas compared to the normal thyroid (C, F, I).

Figure 2.

Quantitative RT-PCR analysis showing relative expression of the EMT markers E-cadherin (ECAD) and ZEB1 in thyroid cell lines. Significantly higher levels of ECAD are expressed in the normal thyroid (NT) cell line (NTHY-UR13-1) compared to the TPC-1 cell line ($p < 0.0005$) and anaplastic thyroid carcinoma (ATC THJ-21T) ($P = 0.035$) cell line. Significantly higher levels of ZEB1 was present in the ATC cell line compared to the PTC ($p = 0.02$) and the normal thyroid ($p = 0.008$) cell lines. Mean $\pm$ SEM. (N= 3 samples for each group).

Figure 3.

Quantitative RT-PCR showing relative expression of Smad7 from total RNA derived from FFPE samples including normal thyroid (NT), follicular thyroid carcinoma (FTC), papillary thyroid carcinoma (PTC) and anaplastic thyroid carcinoma (ATC). Expression of Smad7 is significantly higher in ATC compared to NT ($p=0.01$), FTC ($p<0.05$) and PTC ($p<0.05$). Mean $\pm$ SEM. (N= 3 samples of FFPE tissues for each group from the same blocks used for TMA construction).

Table 1

Patient and Tumor Characteristics of the Thyroid Tumor Cases

Parameter	FA	FTC	PTC	ATC
Number of cases	32	28	57	10
Gender	8M/24F	12M/16F	16M/41F	4M/6F
Age, median (range)	48 (16–77)	55 (18–83)	47 (25–76)	74 (60–86)
Size (mean, st dev), mm	33.5 +/- 14.5	43.9 +/- 25	24.2 +/- 14.1	51.2 +/- 36.1
LN metastases	0	3 (11%)	10 (33.3%)	1 (10%)
Systemic metastasis	0	6 (21%)	1 (1.7%)	4 (40%)

FA-follicular adenoma, FTC-follicular thyroid carcinoma, PTC-papillary thyroid carcinoma, ATC-anaplastic thyroid carcinoma

Table 2

Clinical Outcomes of Patients with Thyroid Tumors

Patient Outcome	FTC	PTC	ATC
Alive without disease	16	44	3
Alive with disease	3	4	0
Died of disease	5	0	5
Died, no evidence of disease	1	0	2
Lost to follow up	3	9	0
Total	28	57	10

FTC-follicular thyroid carcinoma, PTC-papillary thyroid carcinoma, ATC-anaplastic thyroid carcinoma

Table 3

Expression of ZEB1, E-Cadherin, and SMAD7 by immunohistochemistry (positive/total cases)

Antibody	Diagnosis			
	NT	FA	FTC	PTC
ZEB1	0/10 (0%)	0/32 (0%)	1/28 (3.6%)	0/57 (0%)
E-Cadherin	10/10 (100%)	32/32 (100%)	28/28 (100%)	57/57 (100%)
SMAD7 ^a	3/10 (30%)	21/32 (65%)	15/28 (54%)	40/57 (70%)

Immunohistochemical analysis was done on the tissue microarray and quantified as indicated in the Materials and Methods section.

NT-normal thyroid, FA-follicular adenoma, FTC-follicular thyroid carcinoma, PTC-papillary thyroid carcinoma, ATC-anaplastic thyroid carcinoma

^a All cases also had nuclear staining for Smad7. Data showed in Table 3 represent cytoplasmic staining

p<= 0.001 compared to all other groups.