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Original Article

Immunohistochemical detection of epithelial-mesenchymal transition associated with stemness phenotype in anaplastic thyroid carcinoma

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Abstract: Anaplastic thyroid carcinoma (ATC) is a highly aggressive neoplasm resistant to radiation and chemotherapy. Epithelial-mesenchymal transition (EMT) generating cells with stem cell characteristics have been reported to be associated with chemoradioresistance in cultured cells. However, EMT and stem cell properties in ATC have not been fully investigated. In this study, we retrieved 2 thyroidectomy specimens of ATC with coexisting well differentiated thyroid carcinomas (DTCs) including one papillary carcinoma (PTC) and one follicular carcinoma (FTC). We used immunohistochemistry to examine the expression of stem cell markers (nestin, CD133 and CD44) and a marker for EMT (E-cadherin). Intense expressions of nestin, CD133 and CD44, and no expression of E-cadherin were observed in both ATCs. In contrast, the PTC and FTC, and non-neoplastic thyroid tissue in both cases were negative for nestin and positive for E-cadherin. The expressions of CD133 and CD44 were variable in the PTC, FTC, and non-neoplastic thyroid tissue and were at a lower level of expression of these markers in the overall pattern. The results confirmed EMT, demonstrated the stem cell phenotype in ATC, and revealed the difference in expression of these markers between ATC and DTCs/non-neoplastic thyroid tissue. Nestin may be the most specific marker for stemness in ATC by immunohistochemial staining. The results warrant future studies on a large series of cases in order to gain the understanding of the tumor biology and to provide molecular basis for restoring the sensitivities to clinical therapies.

Keywords: Anaplastic thyroid carcinoma, cancer stem cell, epithelial-mesenchymal transition, follicular thyroid carcinoma, immunohistochemistry, papillary thyroid carcinoma

Introduction

Anaplastic thyroid carcinoma (ATC) is a rapidly fatal malignant neoplasm. The overall median survival is 2.5-6 months [1]. Despite advances in the understanding and treatment of other malignancies during the past decades, little improvement has occurred in ATC. Surgery, radiotherapy, and standard chemotherapy do not meaningfully improve survival [2].

It has been observed that differentiated thyroid carcinoma (DTC) affects the risk of developing ATC. Many cases of ATC (from 23% to 78%) have preexisting or coexisting DTCs of follicular cell origin [3-5]. Therefore, it is thought that most ATCs result from dedifferentiation or anaplastic transformation – an intratumoral evolution [6, 7].

Epithelial-mesenchymal transition (EMT) is a dedifferentiation program that converts epithelial cells into a mesenchymal phenotype and is involved in embryogenesis and used pathologically during cancer progression [8]. E-cadherin is a central component of cell junctions and is required for the cell to cell adherens of epithelia [9, 10]. Loss of functional expression of E-cadherin protein and/or transcriptional repression of its mRNA is presently considered as the hall mark of EMT [11]. The consistent absence of E-cadherin expression in ATCs has been reported in the literature [12-14]. Thus, the anaplastic transformation of DTCs is an example of EMT. However, the biologic characteristics of the transformed tumor cells (ATC cells) have

not been fully investigated.

Cancer stem cells (CSCs) have been increasingly evaluated in various tissues and tumors and are thought to be responsible for tumor genesis, recurrence and chemoradioresistance [15-17]. The existence of stem cell-like properties in cancer cells has been demonstrated in hematological cancers and various solid malignant neoplasms [18-20]. However, to our knowledge, there are very few studies of stemness in ATC reported in the literature [21-24]. Among them, only two studies are on the stemness phenotype in ATC. Both studies were cultured cell line-based and the evaluations were based on only one stem cell marker – CD133. Therefore, expressions of stem cell antigens in ATC have not been fully studied.

Identification of specific molecular alterations and mechanisms that underlie this anaplastic transformation is a powerful tool for gaining insight into tumor biology and the discovery of therapeutic targets. In this study, we used immunohistochemistry to examine the expression of multiple markers for stem cell properties and EMT in ATC with comparison with coexisting DTCs (PTC and FTC) and adjacent non-neoplastic thyroid tissue to evaluate the change in the expression profile that occurs during the transformation of DTC into ATC, to find stem cell marker(s) specific for ATC, and to gain understanding of thyroid tumor progression.

Materials and methods

Archival thyroidectomy specimens were obtained from 2 patients with ATC and contiguous DTCs (1 PTC and 1 FTC, respectively) and adjacent non-neoplastic thyroid tissue. Neither of these patients had preoperative treatment for the thyroid tumors. This study protocol was approved by the Institutional Review Board of the University of Texas Health Science Center at Houston.

Immunohistochemical Staining

Immunohistochemial stains were performed on formalin-fixed and paraffin-embedded unstained sections of 4 μm thickness. Four primary antibodies were employed to detect the following antigens: EMT-associated marker: E-Cadherin (ZYMED Laboratories Inc., San Francisco, USA); and stem cell-associated markers: Nestin (Abcam, Cambridge, MA) and CD133 (Abcam) and CD44 (Novocastra, Newcastle upon Tyne, UK).

The slides were stained in an automatic immunostainer using a standard avidin-biotin complex staining procedure. Immunohistochemical reactions were developed with diaminobenzidine as the chromogenic peroxidase substrate, and the slides were counterstained with hematoxylin.

Assessment of Immunohistochemical Staining

Chromogenic signal and subcellular expression pattern were assessed by bright-field microscopy. Both staining intensity and extensiveness were evaluated for each marker. Staining intensity was graded as negative (0), weak (1+), moderate (2+), and strong (3+). Staining extensiveness was the percentage of tumor cells

Table 1. Immunoreactivities for nestin, CD133, CD44, and E-cadherin in ATC, PTC, FTC, and non-neoplastic thyroid tissue

Markers (compartment)	Case 1			Case 2		
	ATC	PTC	NTT	ATC	FTC	NTT
Nestin (cytoplasm)	1-3+ (100%)	±	±	3+ (100%)	0	0
CD133 (cytoplasm)	1-3+ (100%)	1-2+ (100%)	1-2+ (70%)	1-3+ (100%)	1+ (100%)	1+ (80%)
CD 44 (plasmalemma)	1-3+ (95%)	1-2+ (50%)	1+ (5%)	2-3+ (100%)	1+ (30%)	1+ (20%)
E-cadherin (plasmalemma)	0	2+ (100%)	1+ (80%)	0	2+ (100%)	2+ (100%)

ATC: anaplastic thyroid carcinoma; PTC: papillary thyroid carcinoma; FTC: follicular thyroid carcinoma; NTT: non-neoplastic thyroid tissue; %: percentage of cells positively stained; ±: equivocal

positively stained with a range from 0% to 100%. The subcellular expression pattern was evaluated and characterized as: nuclear, cytoplasmic, or plasmalemmal locations.

Results

Microscopic examination showed 2 ATC cases, one with PTC component and the other with FTC transitioning to the ATCs.

Extensive and moderate or strong expressions of cytoplasmic nestin, cytoplasmic CD133, and plasmalemmal CD44, and no expression for E-cadherin were observed in both ATCs. In contrast, the PTC and FTC, and non-neoplastic thyroid tissue in both cases were negative for nestin and positive for E-cadherin (plasmalemmal staining). The expressions of CD133 and CD44 were variable in the PTC, FTC, and non-neoplastic thyroid tissue and were at a lower level of expression of these markers in the overall pattern (intensity and extensiveness) in both cases.

The details of immunostaining results are summarized in **Table 1**. The expressions of these markers are demonstrated in **Figure 1** and **Figure 2**.

Discussion

This study using immunohistochemical staining demonstrates the stemness phenotype in ATC with nestin as the most specific marker and accords with EMT in ATC.

The absence of E-cadherin in ATCs in contrast to the expression of E-cadherin in DTCs and non-neoplastic thyroid tissue in this study is consis-

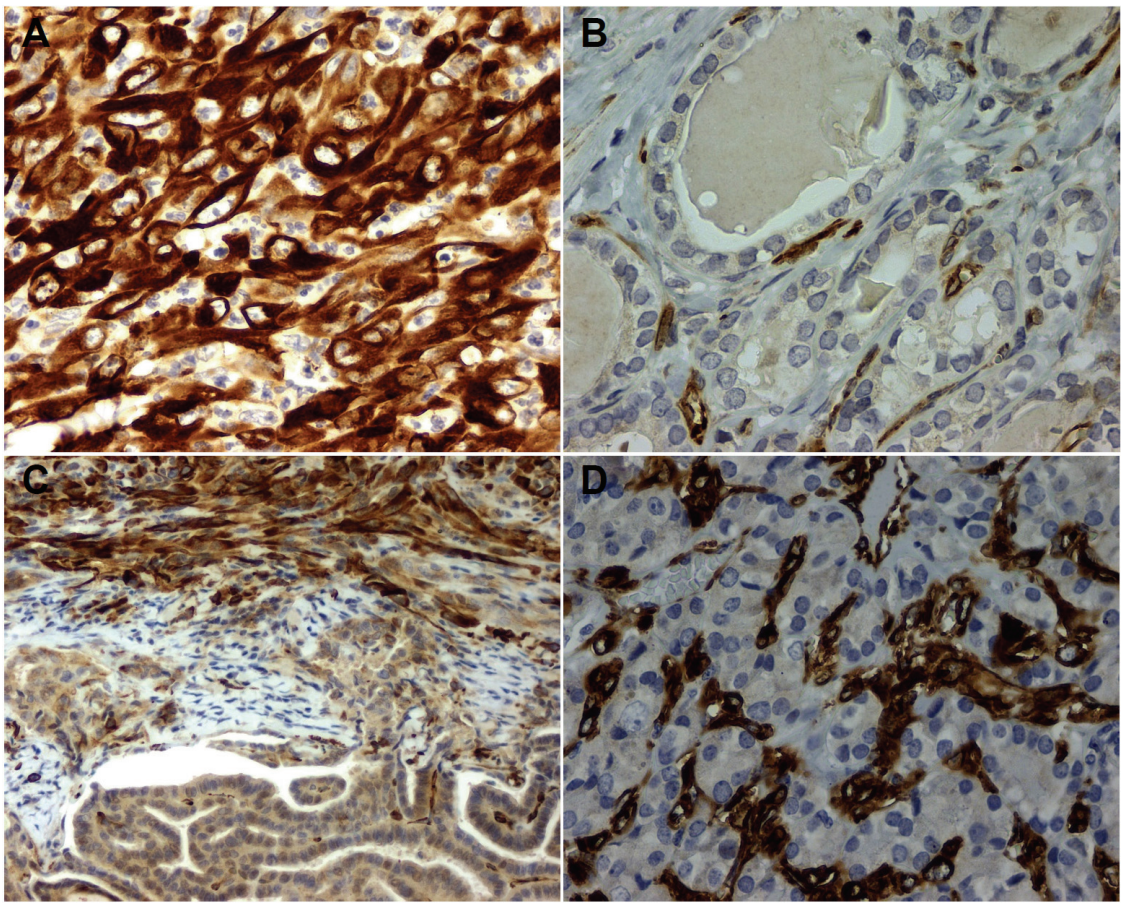

Figure 1. Immunohistochemical staining of nestin in : **A.** anaplastic thyroid carcinoma (Original magnification x 400); **B.** adjacent non-neoplastic thyroid tissue (Original magnification x 400); **C.** papillary thyroid carcinoma component (lower portion)contiguous anaplastic thyroid carcinoma (upper portion) (Original magnification x 200); and **D.** follicular carcinoma (Original magnification x 400).

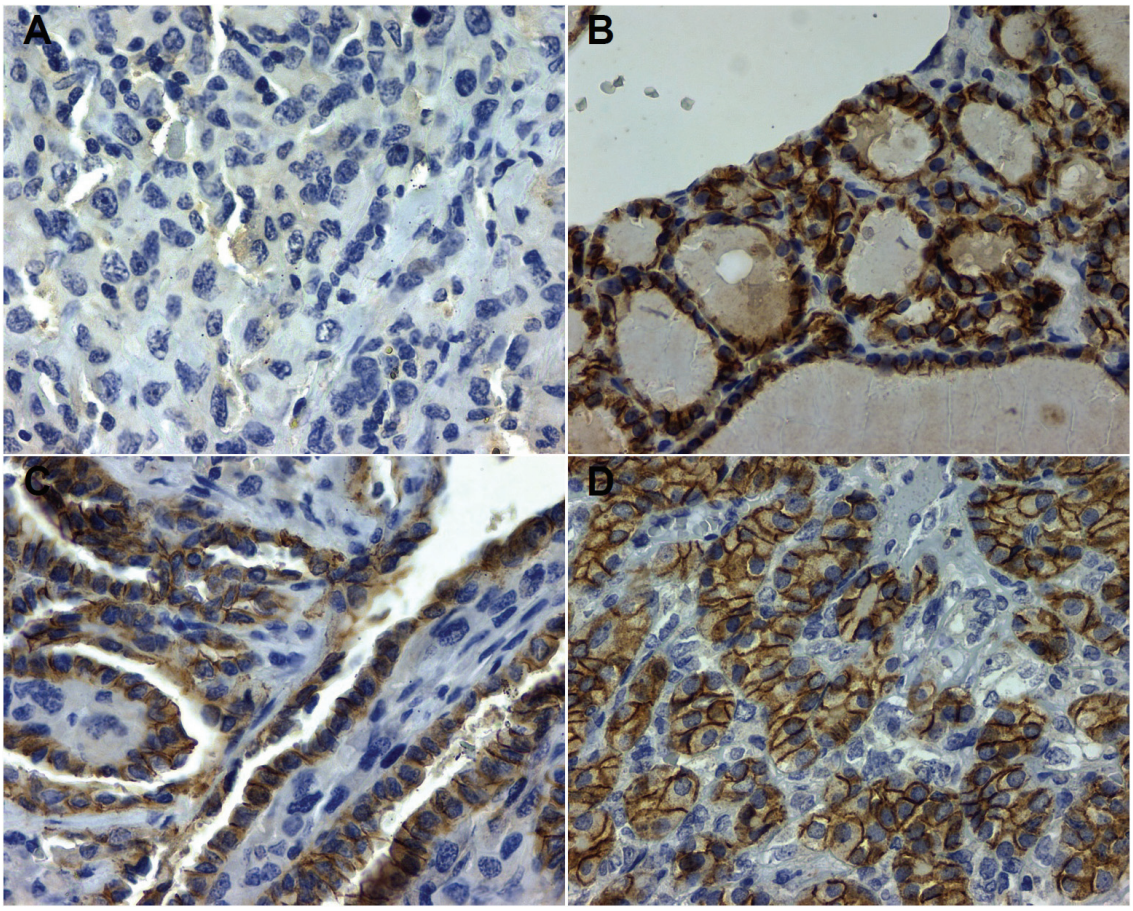

Figure 2. Immunohistochemical staining ofE-cadherin in: **A.** anaplastic thyroid carcinoma (original magnification x 400); **B.** adjacent non-neoplastic thyroid tissue (original magnification x 400); **C.** papillary thyroid carcinoma component (original magnification x 400) and **D.** follicular carcinoma component (original magnification x 400).

tent with previous reports in the literature [12-14] and supports the EMT process from DTC to ATC [25]. The aggressive behavior of ATC accords with the concept that the EMT promotes the invasiveness of the malignant neoplasms [26].

Expressions of several stem cell markers have been identified in various tissues/tumors. However, it is highly debated whether or not these CSCs originate from resident stem cells which progressively acquire a malignant phenotype or from dedifferentiation of mature cells within the organs.

Recent studies have shown evidence suggesting that the two novel concepts, EMT and CSCs, have merged in cancer biology. The studies have consistently demonstrated that EMT cells acquire stem cell characteristics in breast, ovary, pancreas and prostate cancers [27-32]. Mani and co-workers also noted that the maintenance of the stem cell state depends on continuous EMT-inducing signals in cultured cells with EMT [27]. Moreover, it has been shown that EMT and cancer stem cell-like phenotypes facilitate chemoresistence in recurrent ovarian cancer and pancreatic cancer cells [30, 31]. Additionaly recent studies suggest that some important signaling pathways involved in stemness also act as potent EMT inducers in different cellular or biological contexts, e.g., EMT promoting Smad complexes, snail family, Wnt/β-catenin and Notch pathways [33, 34]. Wellner et al also proposed that ZEB1 linked EMT-activation and stemness-maintenance by suppressing stemness-inhibiting microRNAs [35]. Therefore, CSCs may be generated by EMT

of differentiated cells by acquiring specific mutations that endow them with stem cell-like properties [36].

Some of stem cell markers are commonly expressed in multiple organs/tissues/tumors while the others are more organ/tissue/tumor specific.

Nestin is an intermediate filament protein. It was initially found as a stem cell marker in the brain and brain tumors [37]. Lately, nestin expression has been observed in various tissues and tumors and in proliferating vascular endothelial cells. Therefore, nestin is now known as a stem cell marker as well as a novel angiogenesis marker of neovascularization [38]. Nestin expression in tumor cells and vascular endothelial cells has been detected in many types of neurogenic tumors and in various types of epithelial and mesenchymal tumors, e.g., in cultured pancreatic mesenchymal stem/precursor cells [39], triple negative breast carcinoma tissue [40], dermatofibrosarcoma protuberans [41], rhabdomyosarcomas [42], several pediatric tumors in the region of the kidney (Wilms tumor,, mesoblastic nephroma, malignant rhabdoid tumor and desmoplastic small round cell tumor) [43] and in proliferating endothelial cells in colorectal cancer tissue [44]. Moreover, it is reported that nestin expression correlates with tumor malignancy and invasiveness in neurogenic tumors [45] and the nestin-positive populations of tumor cells have a primitive phenotype, which supports the cancer stem cell hypothesis [46].

In our study, only ATC cells express nestin while losing E-cadherin, in contrast to DTCs and non-neoplastic thyroid tissue. This suggests that the anaplastic thyroid carcinoma cells arise via epithelial-mesenchymal transition from differentiated thyroid carcinoma and in the process, gain the phenotype of stemness. There is only one study evaluating nestin in ATC found in the literature [24]. In that study Yamada et al "unexpectedly" found that nestin mRNA was decreased in ATC and increased in differentiated thyroid tumors and normal thyroid cells. One possibility is that the increased nestin protein expression that we observed might not be transcriptional but could be post-transcriptional or post-translational. Further studies are required to elucidate the mechanisms behind these seemingly discordant findings.

Our current study is the first observation that demonstrates nestin antigen expression in ATC by immunohistochemical staining in contrast to its absence in DTCs and adjacent non-neoplastic thyroid tissue. Furthermore, because nestin is also a marker of mesenchymal stem cells [41], its detection in ATC may reflect the acquisition of mesenchymal stemness. Therefore, nestin may serve as a specific stem cell marker for ATC.

CD133 is identified as a stem cell marker in a variety of normal and cancerous tissues. Studies have shown that the expression of CD133 predicted for non-response to chemotherapy in colorectal cancer [47], the survival time of patients with CD133 positive neuroblastoma cells was shorter than that of CD133 negative patients [48] and that CD133 could be a therapeutic target in hepatocellular and gastric cancers [49]. In spite of CD133 as a well known stem cell marker in many other tumors/tissues, there are only two studies of CD133 antigens in ATC has been reported [22,23]. The first study of *in vitro* identification and characterization of CD133+ cancer stem-like cells in ATC cell lines was reported by Zito et al in 2009 [22]. They detected CD133 antigen in cultured ATC cells by flow cytometry and found that the CD133+ cells were more chemoresistant than CD133- ATC cells. Lately, Friedman and co-workers again observed CD133 expression in ATC cell lines and other stem cell properties [23]. The expression of CD133 in patient ATC tissue demonstrated in our current study is consistent with the aforementioned two previous reports in the literature.

In regard to subcellular compartmentalization, the CD133 expression in our current study is predominantly cytoplasmic. The cytoplasmic expression of CD133 has been observed in other types of tumors such as hepatocellular carcinoma and ovarian carcinoma [50, 51]. Moreover, the ovarian cancer patients have poor prognosis if there is diffuse cytoplasmic expression of CD133 [51]. In regard to intensity and extensiveness of CD133 expression, Song et al showed that hepatocellular carcinoma patients with high CD133 expression had shorter overall survival and higher recurrence rates compared to patients with low CD133 expression [52]. Accordingly, in the current study, the ATCs demonstrate more overall expression of

CD133 than DTCs, consistent with a poorer prognosis of ATC than DTCs.

The expression of CD133 in DTCs (PTC and FTC) and at a lower level than that in ATC observed in the current study accords with the study by Mitsutake et al in that a side population with several up-regulated genes related to stemness was identified in PTC (NPA cell line) and FTC (WRO cell line) at a lower level than that in ATC cell lines [21]. However, Friedman and colleagues in their study could not detect CD133 expression in PTC cell lines (both NPA and TPC cell lines) and human normal thyroid cells neighbored to ATCs by immunohistochemistry [23]. Therefore, more future studies are needed to define the specificity of CD133 as a thyroid stem cell marker although the expression of CD133 in ATC likely reflects stemness.

CD44 is identified to be another stem cell marker and its expression was observed in colon, liver, breast, and head and neck cancers [50, 53-55]. CD 44 antigen expression as a stem cell marker in thyroid tissue/tumors has not been reported. This current study identified CD44 expression in thyroid neoplastic and non-neoplastic tissue. The expression in the ATCs is at a higher level than that in the DTCs and non-neoplastic thyroid tissue adjacent to the ATCs. Thus, CD44 may also be one of the stem cell markers in ATC.

The stemness in ATC demonstrated by this study is further supported by the finding of downregulation of stemness-inhibiting microRNAs in ATC cells by Braun J et al [56]. The DTCs (PTC and FTC) and adjacent non-neoplastic thyroid tissue in our study have a certain degree of expression of CD133 and CD44, which suggests that these two markers are not specific for stemness expression in ATC. This is agreed by the observation of Zito et al [22]. They found that two of four ATC cell lines did not express CD133, implicating that the mere presence of CD133 was not sufficient for characterizing the stemness and there must be some other unidentified markers. The current study demonstrates that nestin may be a key marker to indicate the stemness in ATC.

In summary, this is the first study using immunostaining that demonstrates expressions of multiple stem cell markers (nestin, CD133, and CD44) in ATC tissue with comparison to coexisting well differentiated thyroid carcinomas (PTC and FTC) and adjacent non-neoplastic thyroid tissue. Among these three markers, nestin is the most specific one characterizing the stemness of ATC. The expression of stem cell markers with the concomitant loss of E-cadherin is consistent with epithelial mesenchymal transition associated with the stem cell phenotype in ATC. The identification of the properties of cancer stem cells in ATC may be served as a molecular basis for developing targeted therapy for this tumor. The results of our study warrant future studies on a large series of cases.

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