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Twist1 Correlates With Epithelial-Mesenchymal Transition Markers *Fibronectin* and *Vimentin* in Adrenocortical Tumors

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Abstract. *Background/Aim: Although the knowledge regarding adrenocortical carcinomas (ACC) tumorigenesis has significantly improved during the last decade, it still remains to be completely determined. Epithelial–mesenchymal transition (EMT) is a well described transcription factor induced process, postulated as an essential step toward cancer progression and metastasis development. In this context, Twist1 has been described as the EMT master-regulator. The aim of this study was to assess the association among Twist1, fibronectin, vimentin and E-cadherin gene expression in adrenocortical tumor samples. Materials and Methods: Twist1, fibronectin, vimentin and E-cadherin gene expression in 18 adrenal adenomas, 18 ACC, and 24 childhood onset adrenocortical tumors were assessed in formalin-fixed paraffin-embedded tissues. The fold expression was calculated according to the* $2^{\Delta Ct}$ *method. Results: A significant correlation between mRNA levels of Twist1, fibronectin and vimentin was evident. Although their expression was inversely proportional, no association was observed between Twist1 and E-cadherin expression. Conclusion: The expression of Twist1, the major regulator of EMT, is directly correlated to the expression of mesenchymal markers fibronectin and vimentin in ACC samples.*

Adrenocortical carcinoma (ACC) is a very rare disease among the general population, and often presents an aggressive biological behavior (1). Despite a significant increase in the comprehension of the molecular mechanisms responsible for ACC pathogenesis (2), those related to disease progression, such as apoptosis resistance, local invasion, and metastatic process, remain to be completely determined. Epithelial–mesenchymal transition (EMT) process has been described as an essential step toward cancer progression and metastasis development (3, 4). This biological process is regulated by a well-orchestrated integrated system, where the tumoral microenvironment and specific transcription factors play the main role. In this context, basic helix-loop-helix transcription factor *Twist1* has been described as a master regulator of EMT, repressing the expression of epithelial phenotype markers responsible for cell–cell interaction such as *E-cadherin*, and at the same time inducing the expression of mesenchymal genes such as *Vimentin* and *Fibronectin*, which are involved in cancer cell mobility and invasion (5).

The role of EMT in ACC remains to be completely determined. Initially, gene expression analysis reported by Giordano *et al.* (6) described the up-regulation of *Twist1* in ACC compared to benign adrenal tumors. Furthermore, Waldmann *et al.* (7) reported an increased expression of Snail1, another EMT-related transcription factor, in 26 cases of ACC in comparison to 12 cases of adrenal adenomas. Recently, our group originally described that Twist1 protein expression is significantly increased in malignant

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adrenocortical tumors in both adult and childhood onset scenarios (8). Moreover, we found that the protein levels of the EMT marker vimentin were significantly increased in the most aggressive tumors, and that this increase was directly correlated to Twist1 expression. Since then, no additional data have been reported in terms of the role of EMT transcription factors or related markers in ACC.

Materials and Methods

Here we describe a preliminary analysis of *Twist1, Fibronectin, Vimentin* and *E-cadherin* gene expression in 18 adrenal adenomas, 18 ACC, and 24 childhood onset adrenocortical tumors. For this purpose, the following procedures and methodologies were applied: total RNA from formalin-fixed paraffin-embedded (FFPE) tissue samples was obtained using the RNeasy Mini kit (Qiagen, Hilden, Germany) with a previous deparaffinization step, following the manufacturer's instructions. One microgram of RNA was subjected to the DNase Amplification Grade I Kit (Thermo Fisher, Waltham, MA, USA) for removal of DNA contamination and reverse-transcribed into cDNA using the Superscript-III kit (Thermo Fisher). RT-qPCR was performed with SYBR Green Master Mix (Thermo Fisher) in a Rotor-Gene Q (Qiagen, Hilden, Germany). The primers and conditions used to evaluate the mRNA levels of *Twist1, Fibronectin, Vimentin* and *E-cadherin* are described by Pires *et al.* (9). *ACTB* and *B2M* were used as housekeeping genes. The fold expression relatively to control was calculated according to the 2^ΔCt method (10). Spearman rank correlation test was used to correlate numeric continuous *Twist1, Fibronectin, Vimentin* and *E-cadherin* mRNA expression values. A p-value <0.05 was considered statistically significant.

Results

As shown in Figure 1, a significant correlation between *Twist1* and EMT-markers *fibronectin* and *vimentin* was found at the mRNA level. Although their expression was inversely proportional, no association was observed between *Twist1* and *E-cadherin* mRNA levels. These data corroborate our group's previous findings, as we demonstrated low levels of E-cadherin in all types of adrenocortical tumors through immunohistochemistry (8).

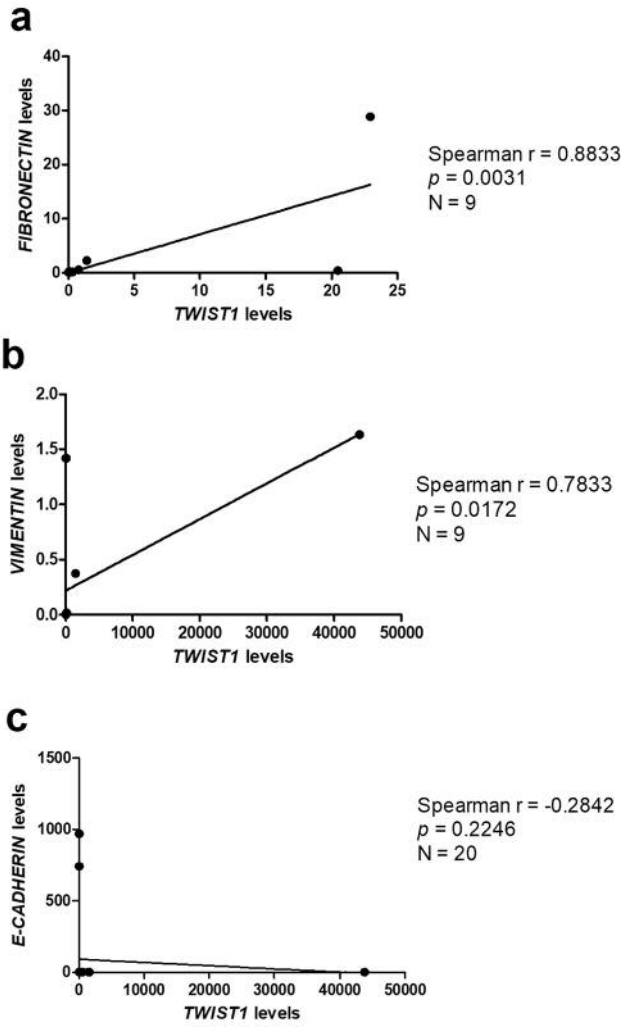

Figure 1. *Spearman correlation analysis of the mRNA expression of Twist1, Vimentin, Fibronectin, and E-cadherin in adrenocortical carcinomas, adenomas and childhood onset adrenocortical tumors samples. a) Twist1 × Fibronectin, b) Twist1 × Vimentin, c) Twist1 × E-cadherin.*

Discussion

The mechanisms by which EMT is activated in ACC are still to be elucidated. However, previous studies have demonstrated the interplay of EMT and well-known tumorigenic pathways in different solid tumors, including the insulin-like growth factor (IGF) (11), Wnt/β-catenin (12), Notch (13), and the Sonic-hedgehog pathways (14). Interestingly, all these pathways have somehow been associated with ACC tumorigenesis and/or progression (2).

The most important pathological functions attributed to *Twist1* in cancer are related to invasion and metastasis by promoting EMT in solid tumors. We observed a positive correlation between *Twist1* and *Fibronectin* and *Twist1* and *Vimentin*, which is in accordance with some reports in different cancer types (15, 16). The regulatory mechanisms that explain these correlations have been previously described by Kwok *et al.* (17), Meng *et al.* (18), and Yang *et al.* (19). Although it was not statistically significant, we observed a negative association between *Twist1* and *E-cadherin* expression, similarly to previously described studies (16, 20). The mechanism behind this finding is the transcriptional repression of *Twist1* on *E-cadherin* promoter, reported originally by Vesuna *et al.* (21).

In summary, the expression of *Twist1*, the major regulator of the EMT biological process, is directly correlated to the expression of the mesenchymal markers *fibronectin* and

vimentin. We emphasize that these are preliminary data and further investigation is needed to consolidate the role of EMT in ACC tumorigenesis.

Ethical Approval

This study was approved by the independent institutional advisory committee in September 24th, 2014 (protocol 33847514.4. 0000.5274). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Conflicts of Interest

The Authors declare that they have no conflict of interest regarding this study.

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