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Specific expression patterns of epithelial to mesenchymal transition factors in gestational molar disease

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ABSTRACT

Introduction: The epithelial to mesenchymal transition, a well-known and re-emerging model in pathology, has not been completely investigated in the field of gestational pathology. This study aims at improving the comprehension of this process in molar disease, even looking for new possible immunohistochemical markers.
Materials and methods: We have analysed the immunohistochemical expression of Twist1 and Snai2, two of the most important transcription factors involved in epithelial to mesenchymal transition, in formalin-fixed paraffin-embedded samples of 23 spontaneous abortive pregnancies, 22 molar pregnancies (10 partial and 12 complete) and 7 term placentas.
Results: Twist1 and Snai2 were highly expressed in stromal villi cells of molar disease. Particularly, Twist1 was highly expressed in complete moles compared to both abortive pregnancies ($p < 0.001$) and partial moles ($p < 0.05$). Also Snai2 was more expressed by complete moles, differentiating them from non-molar abortions ($p < 0.05$).
Discussion: On the basis of the known cadherins and claudins expression in these pathologies, our new findings reinforce the hypothesis of the involvement of epithelial to mesenchymal transition in early molar pregnancies and above all in complete moles. Furthermore, we highlighted that in molar disease not only the trophoblast, but even the villi stromal cells, are involved. Thanks to their specificity, furthermore, these Twist1 and Snai2 could be used as additional immunohistochemical tool in the diagnosis of complete molar disease, with Twist1 as the first choice.
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1. Introduction

Epithelial to mesenchymal transition (EMT) is a process in which the epithelial elements lose their polarity and cell-to-cell contacts, undergo remodelling of the cytoskeleton with morphological modifications, and acquire migratory capacity [1]. Particularly, in neoplastic pathology EMT is an important mechanism for the onset and metastasization of many cancer types [2–12].

The down-regulation of cadherins, a family of cell-surface adherence junctional proteins, represents a fundamental step of EMT. They are mediators of cell-to-cell adhesion in epithelial tissues and regulators even of trophoblast cell behaviour in the placental development. With the reduction of cadherins expression, the trophoblast acquires a mesenchymal phenotype characterized by proliferative and invasive capacity; this eases colonisation of the maternal uterine wall and fetal–maternal interaction [13]. The most important cadherin involved in this process is the E-cadherin: its aberrant expression, as detected by immunohistochemistry (IHC), has been associated with invasive molar pregnancy, where invasive moles displayed significantly lower E-cadherin expression than benign mole [14,15].

Several cytokines, triggering the down-regulation of E-cadherin, lead consequently to EMT in different types of cancer [1,2,16–21]. Most of these signalling molecules promote E-cadherin repression through the modulation of a set of pleiotropically acting

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transcription factors, including members of Snail (Snai1, previously known as Snail and Snai2, previously known as Slug) and basic helix-loop-helix (E47 and Twist1, previously known as Twist) families [1,18]. Beyond their effects on cadherins, they could potentially activate the transcription of genes characteristic of the mesenchymal state, like vimentin and fibronectin, inducing EMT. Twist1 is indicated as an essential protein for trophoblastic differentiation, proper gastrulation, mesoderm formation and neural crest migration [22–28]. Furthermore, it can mediate EMT during cancer progression, particularly in the acquisition of invasive and metastatic potential, and can be expressed also in carcinosarcomas [29]. Snai2 has similar features; moreover, in tumour cells, as well as it is correlated with cadherin down-regulation, Snai2 enhances tumour cell proliferation and invasiveness even through cyclin D1 [11,23,30–35]. In a recent study it has been investigated the expression of some EMT markers in placental development and disease by IHC, indicating claudin-4 as a marker connected to placental pathologies, and Twist1 as an important factor in placental development [36].

Our study, the first focused on the initial gestational trimester and that considers separately complete and partial moles, has as aim studying further EMT markers in molar disease, looking for possible immunohistochemical applications in differential diagnosis.

2. Methods

This study was conducted in accordance with the Good Practice guidelines, the Declaration of Helsinki and Italy's laws, ethics and regulations, and was approved by the Ethical Committee (15/n.p.720/prog.652). Cases of abortive and molar pregnancies were retrieved from the archives of Pathology Unit of Verona University Hospital. As inclusion criterion, the gestational age had to be comprised between 6 and 12 weeks, better to represent the early abortive pathology. Gestational age was calculated for each from the last normal menstrual period to the date of curettage, and/or using data from clinical history, clinical evaluation, ultrasound findings and laboratory tests (particularly: beta-hCG).

We selected a total of 45 cases classified as follows: 23 spontaneous abortive pregnancies (SAP), 12 of which had clearly occurred embryogenesis (AE) as ascertained by the presence of fetal tissue, and 11 without clear signs of embryogenesis (ANE); 10 partial molar pregnancies (PM), seven of which with clear signs of embryogenesis, and 12 complete molar pregnancies (CM). Among the 11 ANE, to test the feasibility of immunohistochemistry (IHC) even on necrotic tissue, 4 cases with massive necrotic phenomena (ANE-Ne) were included. The selected cases are grouped on the basis of disease and gestational age in Table 1. For each category, we have selected the last diagnosed cases in our Hospital in a chronological continuity. Selection of molar pregnancies, furthermore, was limited to cases with IHC for p57 and ploidy analysis available and consistent with molar diagnosis, as well as for beta-hGC values. Since EMT is described as a dynamic process in placental development [13], Twist1 and Snai2 immunolabelling was also performed on 7 term placentas. Hematoxylin-eosin sections were reviewed and, for each case, the most representative inclusion was chosen.

Table 1
The abortive cases investigated in this study are here grouped on the basis of the type of disease and weeks of gestation (AE: spontaneous abortions with clear occurred embryogenesis, ANE: spontaneous abortions without clear signs of embryogenesis, ANE-Ne: ANE with massive necrotic phenomena, PM: partial moles, CM: complete moles).

Weeks	Cases	AE	ANE	ANE-Ne	PM	CM
6	5	1	1	1	0	2
7	7	1	0	0	3	3
8	13	3	3	0	3	4
9	12	5	2	1	2	2
10	4	0	1	1	1	1
11	3	2	0	0	1	0
12	1	0	0	1	0	0
Total	45	12	7	4	10	12

Immunohistochemistry was performed with anti-Twist1 (clone: Twist2C1a, 1:80 diluition, Santacruz/USA) and anti-Snai2 (clone: rabbit, 1:350 dilution, Xeptagen/Italy) antibodies, using 4 μm formalin-fixed paraffin-embedded sections. Heat-induced antigen retrieval for Twist1 and Snai2 was performed using a heated plate and 0.01 mol/l of citrate buffer, pH 8.9, for 15 min. Only for Snai2, the antigen–antibody reaction was incubated overnight at 4 °C. Light nuclear counterstaining was performed with hematoxylin. All samples were processed using a sensitive peroxidase-based 'Bond polymer Refine' detection system in an automated Bond immunohistochemistry instrument (Vision-Biosystem, Leica, Milan, Italy). Sections incubated without the primary antibody served as negative controls. Tissues were considered positive only if nuclear expression was present.

Firstly, in the immunohistochemical evaluation, on the whole section area the percentages of nuclei immunolabeled by Twist1 and Snai2 IHC were calculated. The immune reactivity was assessed separately in: stromal villi cells, endothelial cells of the villi vessels and trophoblastic cells. Then, a qualitative evaluation was done, assigning a score on the basis of the staining intensity as follows: score 0 = no staining, score 33 = weak nuclear staining (+), score 66 = moderate nuclear staining (++), and score 100 = strong nuclear staining (+++). Lastly, a comprehensive score was calculated by averaging between percentages of immunolabeled cells and staining intensity. For example, in a case in which we recorded a percentage of 80% of immunolabeled stromal villi cells by Twist1, and in which the intensity of the reaction was moderate (i.e. ++, score 66), we obtained the comprehensive score by calculating (80 + 66)/2 = 73. The immunostaining was evaluated separately and in blind by two surgical pathologists (C.L., E.M.). In 4 cases there was disagreement. Particularly, there was disagreement in 3 cases on the percentage of positive cells (case 1, CM: Twist1 stromal positive cell: 80%(C.L.) vs 70%(E.M.); case 2, ANE: Twist1 trophoblast positive cell: 50%(C.L.) vs 60%(E.M.); case 3, AE: Snai2 stromal positive cell: 20%(C.L.) vs 10%(E.M.)) and in one case on the intensity score of staining (case 4, AE: Snai2 intensity score of staining: 66(C.L.) vs 33(E.M.)) In the 4 cases in which there was disagreement, other two surgical pathologists (N.A., A.R.) watched separately and in blind the slides (case 1: 70%(A.N.), 70%(A.R.); case 2: 60%(A.N), 60%(A.R.); case 3: 10%(A.N.), 10%(A.R.); case 4: 66(A.N.), 66(A.R.)). The final values, used in this paper for these 4 cases, were the values selected by the majority (3 on 4) of the pathologists (case 1: 70%, case 2: 60%, case 3: 10%, case 4: 66). A final agreement on the selected values was obtained watching at a multi-headed microscope these 4 cases.

2.1. Statistical analysis

All statistical analyses were performed using GraphPad Prism 5.04 for Windows (GraphPad Software, San Diego, California, USA) and R (R Foundation for Statistical Computing, Vienna, Austria). After testing for normal distribution of the data by both Shapiro–Wilk and D'Agostino–Pearson test, the scores of the immunohistochemical markers among different types of category (SAP, PM and CM) were analysed by Kruskal–Wallis test and post-hoc, pairwise comparisons were assessed by Dunn's test. These results were visualized by box- and -whisker plot. Furthermore, to detail

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