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ORIGINAL ARTICLE

Identification of epithelial-mesenchymal transition markers (EMT) by immunohistochemistry in pediatric osteosarcoma and association with clinical outcomes

Identificación de marcadores de transición epitelio-mesénquima (TEM) por inmunohistoquímica en osteosarcoma pediátrico y asociación con desenlaces clínicos

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What do we know about the subject matter of this study?

In osteosarcomas, the biological mechanisms associated with their clinical behavior have not been clarified and the epithelial-mesenchymal transition has been proposed as a mechanism that may explain their clinical aggressiveness.

What does this study contribute to what is already known?

In this work, we identified by immunohistochemistry two transcription factors involved in the epithelial-mesenchymal transition in pediatric osteosarcoma samples; these results suggest that this type of tumor cells can use this mechanism to spread and invade other tissues.

Abstract

The epithelial-mesenchymal transition (EMT) is the ability of epithelial and mesenchymal cells to exchange phenotypes transiently. Its identification in carcinomatous cells has been associated with aggressive clinical phenotypes. In sarcomas, this ability is under study. **Objective:** to evaluate the expression of two transcription factors involved in EMT by immunohistochemistry in pediatric osteosarcoma and its association with clinical outcomes. **Patients and Method:** A retrospective cohort study in children under 18 years of age with osteosarcoma diagnosis. Immunohistochemistry was performed for Snail and Twist-1 expressions from samples collected at the time of diagnosis. Corre-

Keywords:
Transcription Factors; Snail Family; Twist-Related Protein 1; Osteosarcoma; Epithelial-Mesenchymal Transition

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lations between immunohistochemistry and the clinical outcomes and overall survival were performed. **Results:** 53 patients were included. There were 26 positive cytoplasmic cases (49.1%) in Snail expression and were correlated with the presence of multiple metastases ($p = 0.02$) and distant bone metastases ($p = 0.01$). On the other hand, 45 cases (84.9%) were positive in Twist-1 expression in the nuclear location, showing no association with the analyzed clinical variables. **Conclusions:** Snail and Twist-1 were frequently expressed in pediatric cases of osteosarcoma. Cytoplasmic Snail was correlated with the presence of multiple metastatic disease and distant bone metastases. The positivity of both markers suggests the activation of these proteins as regulators of EMT events in this tumor, suggesting a role in the phenomena related to the clinical presentation of the disease.

Introduction

Epithelial-mesenchymal transition (EMT) is a biological event where epithelial cells adopt characteristics of mesenchymal cells, and its reverse process is the mesenchymal-epithelial transition (MET) which means that mesenchymal cells behave like epithelial cells. These phenotypic exchanges are reversible, and the cells are capable to reacquire their original phenotype[1]; both are essential processes in physiological phenomena such as embryogenesis, organogenesis, and healing[2].

The EMT/MET processes have been identified in tumor cells of epithelial origin that acquire mesenchymal properties which give them the ability to extravasate, migrate, and invade tissues distant from the primary tumor, qualities that have been associated with more aggressive clinical presentations and poor prognostic outcomes[3]. Sarcomas also seem to present this type of cellular plasticity, presenting an inverse behavior where there is a transition from mesenchymal cell to epithelial phenotype, which also seems to be associated with a greater aggressiveness of the disease[4].

The most frequent sarcoma in the pediatric age group is osteosarcoma, a primary bone tumor with an incidence ranging from 3 to 5 cases per million in males and 2 to 4 cases per million in females[5]. To date, the biological mechanisms underlying the aggressive behavior of osteosarcomas have not been fully clarified; some studies indicate that this type of tumor may undergo phenotypic changes involved in the EMT and MET which may have implications for aggressiveness and clinical outcomes[6-8].

EMT/MET events are mediated by a group of activating transcription factors whose basic set includes Snail1 (Snail), Snail2 (Slug), Twist-1, Twist-2, Zeb1, and Zeb2, which are molecules that regulate both biological phenomena and activation in tumor cells[9,10].

In addition to regulating EMT/MET processes during the embryonic period and organogenesis, the overexpression of Snail has been observed in several carcinomas, where it has been correlated with a higher frequency of metastasis and greater aggressiveness in the clinical presentation[11], and with chemoresistance, mainly to cisplatin, doxorubicin, and 5-fluorouracil in breast and ovarian cancer[12].

On the other hand, Twist-1 is capable to repress or induce the expression of proteins related to the regulation of embryogenesis and is fundamental in the regulation of cellular changes, such as the invadopodia formation and the induction of vasculogenic mimicry[10]. Several investigations have shown that Twist-1 expression is associated with unfavorable clinical outcomes and increased frequency of metastasis in several types of solid tumors, including prostate, cervix, breast, stomach, and pancreas ones[13].

The objective of this study is to evaluate Snail and Twist-1 expression by immunohistochemistry (IHC) in osteosarcomas in pediatric patients at diagnosis, and a possible correlation of positivity with clinical behavior, chemotherapy response and toxicity, and patient survival is studied.

Patients and Method

A cohort, retrospective study was performed on pediatric patients with confirmed diagnosis of osteosarcoma treated in a national referral institution in Bogota, Colombia, between 2012 and May 2020. To determine the size of the population to be included, a non-probabilistic sampling by convenience was performed since it was not possible to estimate the proportion of cases that had the necessary biological material to perform the IHC studies. Therefore, all patients with a confirmed diagnosis of osteosarcoma who had a kerosene tissue block and hematoxylin-eosin-stained tissue slides of the initial diagnosis for the IHC study were included. Cases in which there were limitations with the clinical records due to lack of follow-up or abandonment of treatment at the institution were excluded.

Demographic, clinical, and outcome variables were analyzed. Among the clinical variables, the following

were recorded: location of the lesion, stage according to Enneking classification, presence and location of metastases at diagnosis, and response to chemotherapy measured as percentage (%) of necrosis after neoadjuvant therapy. In addition, toxicity events associated with chemotherapy were recorded in the two periods (neoadjuvant and adjuvant), and variables of hematologic, cardiac, and renal toxicity and the presence of mucositis were included. The outcome variables were relapse or progression of the disease, overall survival, and the development of second neoplasms.

For the histopathology study, after identification of the cases, the biological samples (paraffin block and IHC study) were reviewed and those with the highest tumor representativeness were selected for processing with Snail and Twist-1 antibodies.

Formalin-fixed paraffin-embedded samples were cut at 4 microns into 6 loaded slides for each case and then deparaffinized in xylene and dehydrated in ethanol, followed by citrate buffer retrieval for 10 minutes and inactivation of endogenous peroxidase with 3% H2O2 for 20 minutes. Subsequently, the samples were incubated with primary antibodies against Snail and Twist-1 (Novusbio®) for one hour. Each tissue was then washed for 1 minute with phosphate-buffered saline. Then, the slides were incubated with the secondary antibody marked with horseradish peroxidase at 37°C for 30 minutes and visualized by incubation with DAB solution (Sangon Biotech®, Shanghai, China). Subsequently, the slides were stained with hematoxylin for 1 minute and washed for 3 minutes with water. Finally, the slices were dehydrated with ethanol followed by xylol and mounted with resin and fixed on a slide.

The IHC study was read as follows: not informative in case of scanty material, artifact, or tumor necrosis; negative: < 5% of tumor cells, focal: between 5-50% of tumor cells, and diffuse: between 50-100% of tumor cells. Staining intensity was considered weak if only observed at 40X, moderate if observable at 10X, or strong if observed at 4X, and according to the localization as cytoplasmic, nuclear, or membranous. All cases were evaluated by two pathologists with experience in pediatric pathology and differences were resolved by consensus.

Baseline processing of the variables was performed in IBM Statistical Package for the Social Sciences 22 system (IBM Corp., Armonk, NY). Descriptive analysis was performed for quantitative variables in their measures of central tendency and nominal variables in absolute and relative frequencies.

Descriptive IHC characterization of the cohort was performed. Cases that were considered positive (defined as mild staining based on intensity) for each transcription factor were statistically correlated with clinical characteristics using the chi-square test and correlation with overall survival was assessed using the Kaplan-Meier method with the log-rank test. The results were interpreted based on a statistical significance level of 0.05.

This work was approved by the ethics committee of the institution according to CEI 75-17 No. 007.

Results

General characteristics of the cohort

In the period evaluated, 53 patients met the inclusion criteria. Of these, 33 cases (62.2%) were male. The median age was 13 years (IQR 3-17). The most frequent location of the primary tumor was the femur in 29 patients (54.7%), followed by the tibia in 14 cases (26.4%), humerus in 8 patients (15.1%), and fibula and iliac crest each with 1 patient (1.9%). In 51 patients (96.2%), the histological classification of osteosarcoma was of conventional type; one case of small-cell osteosarcoma (1.9%), and 1 case (1.9%) of telangiectatic type. Metastases were found in 18 patients (34%) at diagnosis.

The treatment characteristics of the cohort were as follows: 27 patients (50.9%) received high-dose methotrexate therapy, 23 patients (43.4%) were managed according to the 2017 ACHOP protocol (Ifosfamide, Etoposide, Doxorubicin), and 3 cases (5.7%) received both therapy schemes combined. 48 patients (90.5%) underwent local disease control surgery and only 1 patient (2.8%) achieved positive resection margins. In 3 patients (5.6%), metastasectomy was performed as first-line therapy. The mean necrosis % of the entire cohort was 67.8% (SD 34.4); of the total cohort, 20 patients (37.7%) had necrosis greater than 90% after neoadjuvant chemotherapy. First-line radiotherapy was not used in any of the cases.

During follow-up, there were 10 patients (18.9%) with disease progression and 9 cases (16.9%) with disease relapse after completing the treatment, and 15 patients (28.3%) died of different causes: 7 (46.6%) due to progression of the osteosarcoma, 4 (26.6%) due to relapsed disease, 3 (20%) due to second neoplasms, and 1 case (6.6%) due to complications derived from therapy.

Immunohistochemistry results

Snail

More frequent Immunostaining was found in the cytoplasmic location with 26 cases (49.1%); of these, 14 cases (53.8%) had focal distribution and the remaining 12 cases (46.1%) had diffuse distribution; regarding the intensity of staining, it was distributed as follows: 7 cases (26.9%) with strong staining, 7 cases (26.9%)

moderate, and 12 cases (46.1%) with weak staining. In 26 cases (49.0%), no Snail positivity was observed in the sample at any location. Table 1 presents the clinical variables of the cytoplasmic Snail-positive cases and their correlation. A statistical association was found between positive and strong cytoplasmic Snail positivity and the presence of multiple metastases at disease onset (p = 0.02) and with bone localization in metastatic disease (p = 0.01).

Only two cases (3.8%) showed immunostaining in the nucleus, both had diffuse distribution, and the intensity was moderate. The first of these cases was a 12-years-old female patient, with pathologic fracture at the onset, the location of the tumor was femoral, and without metastasis at diagnosis; she received neoadjuvant, salvage surgery and the degree of post-chemotherapy necrosis was 25%; however, during treatment, she presented progression of the disease at the pulmonary level which caused her death. The second case, another female patient, 13 years old, with primary location of the tumor in the femur, without metastasis at the time of diagnosis, received neoadjuvant, salvage surgery and the degree of post-chemotherapy necrosis was 89%; the patient had a relapse in the first year of follow-up after the end of treatment.

Given the high frequency of positivity in the cytoplasm of this marker, it was decided to perform an analysis of overall survival using the Kaplan-Meier estimate of osteosarcoma cases with positive Snail at the cytoplasmic level, finding no significant differences between those who had positive immunostaining and the group with negative immunostaining and overall survival (Figure 1).

Twist-1

In 12 cases (22.5%), immunostaining was observed in the cytoplasm; of these, in 8 cases (66.6%) the marker localization was diffuse throughout the sample and the 4 cases (33.3%) had focal localization. Regarding staining intensity, there were 5 cases (41.6%) with weak, 5 cases (41.6%) with moderate, and 2 cases (16.6%) with strong staining. . No statistical association was observed between cytoplasmic Twist positivity and the clinical variables studied.

Nuclear Twist was found in 45 cases (84.9%); of these, 31 cases (68.8%) had diffuse localization and 14 cases (31.1%) had focal localization. In relation to the staining intensity, 15 cases (33.3%) presented weak staining, 17 cases (37.7%) moderate, and 13 cases (28.8%) strong staining; 10 cases had nuclear and cytoplasmic staining at the same time. For Twist-1, 6 cases (11.3%) did not present immunostaining. Table 2 shows the summary of clinical features and nuclear staining of Twist-1.

Table 1. Cytoplasmic Snail expression and correlation with clinical features

Clinical features	Snail n (%)		p value	Cases according with to the amount of positively stained cells			p value
	- n = 26	+ n = 26		+ (n = 12)	++ (n = 7)	+++ (n = 7)	
Prensece of metastasis	11 (42.3)	7 (26.9)	0.38	4	1	2	0.53
Metastasis							
Only site	11 (42.3)	4 (15.4)		3	1	-	
Multi-site	-	3 (11.5)	0.04*	1	-	2	0.02*
Location of metastases							
Skip metastasis	3 (11.5)	3 (11.5)	0.62	3	-	-	0.43
Lungs	8 (30.7)	3 (11.5)	0.33	1	1	-	0.64
Distant bones	-	4	0.06	2	-	2	0.01*
Relapse disease	22 (84.4)	4 (15.4)	0.52	3	1	-	0.24
Disease progression	22 (84.4)	4 (15.4)	0.72	3	-	1	0.65
Tumor necrosis rate > 90% after neoadjuvant chemotherapy	10 (38.5)	10 (38.5)	0.76	3	4	3	0.39
Hematologic toxicity	4 (15.4)	22 (84.6)	0.50	12	5	5	0.59
Liver toxicity	17 (65.4)	9 (34.6)	0.27	5	3	1	0.35
Kidney toxicity	25 (96.2)	1 (3.8)	0.74	-	1	-	0.74
Cardiac toxicity	24 (92.3)	2 (7.7)	0.68	1	-	1	0.45
Mucositis	24 (92.3)	2 (7.7)	0.51	1	1	-	0.47
Second Neoplasms	24 (92.3)	2 (7.7)	0.57	-	1	1	0.31

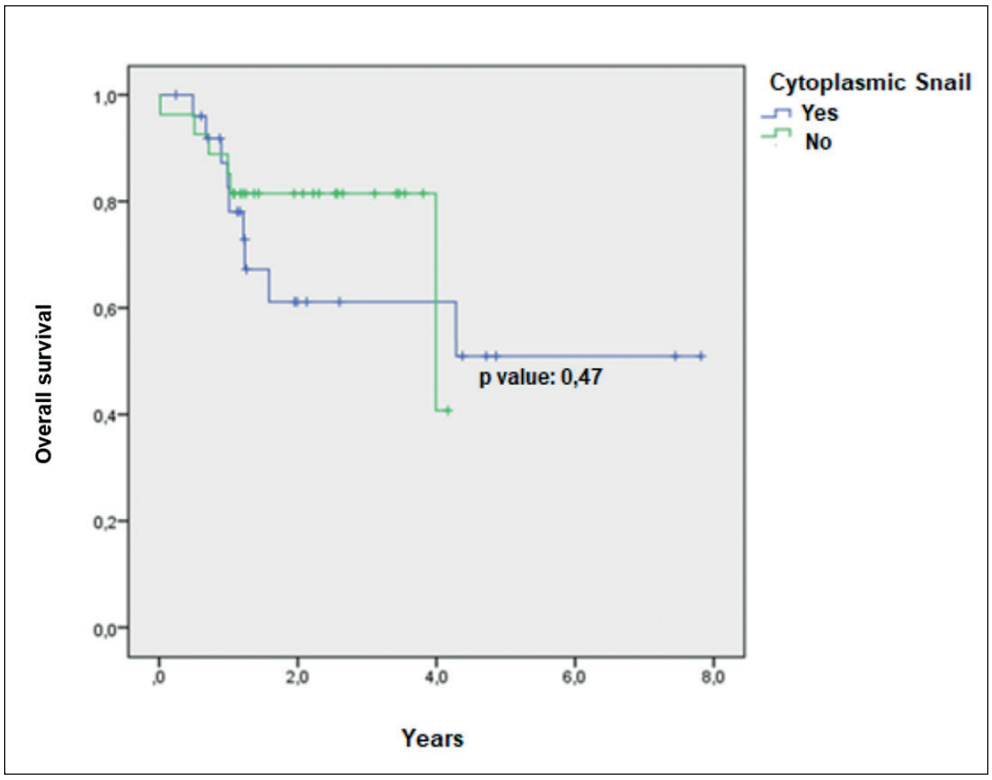

Figure 1. Overall survival and cytoplasmic Snail**.**

Table 2 . Nuclear Twist-1 expression and correlation with clinical features

Variable	Twist n (%)		p value	Cases according to the amount of positively stained cells			p value
	- (n = 6)	+ (n = 45)		+ (n = 15)	++ (n = 17)	+++ (n = 13)	
Presence of metastasis	1	17	0.16	7	6	4	0.24
Metastasis							
Only site	1	14		6	5	3	
Multi-site		3	0.83	1	1	1	0.83
Location of metastases							
Skip metastasis	1	6	0.66	3	2	1	0.66
Lungs	1	10	0.61	5	4	1	0.61
Distant bones	-	4	0.85	1	1	2	0.85
Relapse disease	1	8	0.58	3	5	-	0.58
Disease progression	1	9	0.52	3	4	2	0.52
Tumor necrosis rate > 90% after neoadjuvant chemotherapy	4	16	0.35	8	3	5	0.35
Hematologic toxicity	3	37	0.34	12	12	13	0.20
Liver toxicity	1	21	0.44	7	8	6	0.22
Kidney toxicity	-	2	0.71	-	-	2	0.71
Cardiac toxicity	-	4	0.50	1	2	1	0.50
Mucositis	1	4	0.57	1	3	-	0.57
Second Neoplasms	1	2	0.40	-	-	-	-

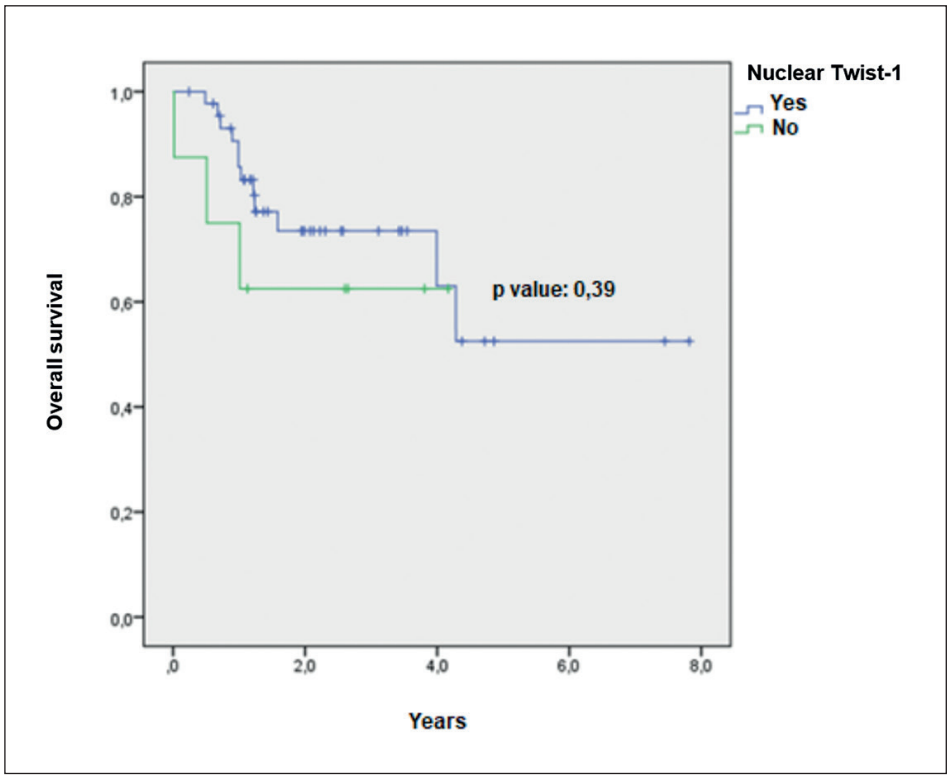

Figure 2. Overall survival and nuclear Twist-1.

There was no correlation between nuclear immunostaining positivity and the variables presence or type of metastasis, relapse, disease progression, response to chemotherapy, or any type of chemotherapy-related toxicity.

The analysis of overall and event-free survival using the Kaplan-Meier method was performed for osteosarcoma cases with Twist-1 positive at the nuclear level, finding no significant differences among those with positive immunostaining (Figure 2).

The two cases that showed Snail staining at the nuclear level also presented Twist-1 nuclear positivity.

Figure 3 shows the imaging findings in the IHC of Snail and Twist-1.

Discussion

Cellular plasticity regulated by transcription factors such as Twist-1, Snail, and others such as Slug, ZEB1, and ZEB2 plays a fundamental role in regulating the transition between the epithelial and mesenchymal state[14-16]. This investigation evaluated the expression by IHC of Snail and Twist-1 in pediatric osteosarcomas and assessed the correlation of this expression with different clinical variables and outcomes.

Since Snail and Twist-1 are transcription factors, it was expected that the staining would be found primarily in the nucleus to be considered positive and correlated with the activity of each of these molecules, however, the locations varied for both markers.

In the case of Snail, the nuclear localization was infrequent and only two patients presented this immunostaining pattern, however, it is striking that they share clinical characteristics such as age, sex, and disease onset without metastasis and, despite receiving treatment, both patients also had relapses. Although it corresponds to only two cases of the cohort evaluated and it is not possible to generalize due to the size of the sample, it could be suggested that the nuclear expression of Snail evaluated by IHC is a factor that can predict the presence of relapses.

Additionally, it was found that up to 49% of the cases have positive Snail staining with cytoplasmic localization. It was decided to explore the correlation between clinical and outcome variables given the significant positivity in the cohort and, strikingly, there was a statistical correlation between the presence of multiple metastatic sites at diagnosis and the presence of bone metastases distant from the primary tumor site and this association was accentuated with strong Snail positivity. Other authors have reported cytoplasmic expression by IHC of Snail in tumors also of mesenchymal origin, and it is presumed that this location could be related to a type of post-transcriptional regulatory level of cell growth and differentiation[17].

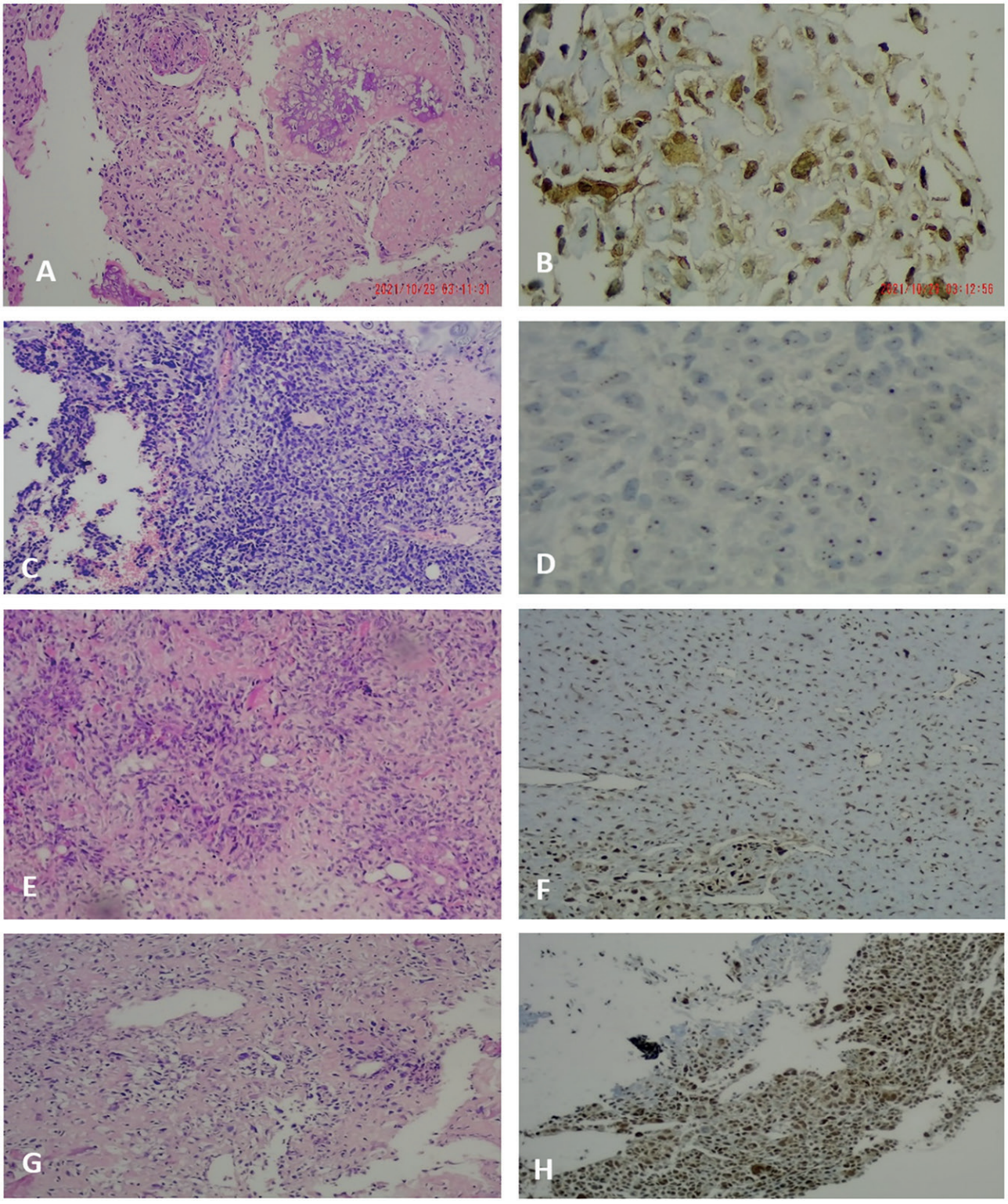

Figure 3. *Snail and Twist-1 by immunohistochemistry (IHC). Snail.* The image shows a conventional osteosarcoma **(a)** positive for cytoplasmic staining of Snail in tumor cells **(b)**. Hypercellular osteosarcoma with less osteoid formation **(c)** shows nuclear dot expression for Snail **(d)**. *Twist-1*. Conventional osteosarcoma is observed **(e)**, positive for nuclear Twist-1 by IHC **(f)** it is highlighted that the nuclei are embedded in the osteoid stroma. Another similar osteosarcoma **(g)** in which strong nuclear positive Twist-1 is also seen **(h)**. In this case, the stroma is less abundant.

Snail has already been associated in other studies as a factor that can predict disease progression when it is overexpressed in osteosarcomas due to its capacity to regulate molecules such as E-cadherin[18], however, cytoplasmic staining has not been previously correlated with clinical outcomes, therefore, this finding deserves attention because it could be a surrogate expression of EMT/MET and that eventually could function as an early marker of poor prognosis, although further research is needed.

Twist-1 is a fundamental transcription factor in the regulation of EMT/MET and current evidence shows that its expression in tumor cells favors the dissemination of the primary tumor and its subsequent establishment in distant organs[10,13]. Strikingly, in this work, we found a frequent expression of Twist-1 at the nuclear level, reaching almost 85% of the cases evaluated, however, contrary to what was documented by Ying et al.[19], we could not find an association of this expression with factors such as the presence of metastasis or overall survival.

As transcription factors, Snail and Twist need to enter the nucleus to fulfill their function, however, it is possible that the transport systems to the nucleus, importins and exportins, which must be intact for their mobility, present some type of alteration in this type of tumors. On the other hand, Snail is an unstable protein that degrades rapidly. It is also possible that, in these cases, some post-transcriptional modification (phosphorylation, ubiquitination, and lysine oxidation) increases the stability of the protein in the cytoplasm. Both biological mechanisms should be explored in further studies[20,21].

Although EMT/MET-related transcription factors have also been associated with chemoresistance and response to chemotherapy[11,12,22,23], in this investigation no association was found with necrosis % post neoadjuvant nor with therapy-related toxicity.

Among the limitations, we can mention that it is a single-center study with a limited number of samples, only two markers related to EMT/MET events were explored, and the tool used was IHC. Other structural aspects of both proteins could be explored to confirm or rule out our findings as described in the literature and, although our results with Twist analysis were inconclusive, it has been implicated as a prognostic marker in osteosarcoma in other investigations[24-26].

In conclusion, Snail and Twist-1 can be expressed in different subcellular locations and frequently in a sample of pediatric patients with osteosarcoma. The most striking finding of this study was the correlation between the presence of positive cytoplasmic Snail and the presence of multi-site metastatic disease. Snail may participate in migration and metastasis-facilitating events in pediatric osteosarcoma, suggesting an essential role in phenomena related to the clinical aggressiveness of the disease. We suggest that the positivity of these proteins through IHC is related to their activation and possible induction of EMT/EMT events in these tumor cells, however, we failed to demonstrate a clear role in correlation with the different clinical events or patient outcomes in this cohort. Further studies are required to confirm or rule out our findings.

Ethical Responsibilities

Human Beings and animals protection: Disclosure the authors state that the procedures were followed according to the Declaration of Helsinki and the World Medical Association regarding human experimentation developed for the medical community.

Data confidentiality: The authors state that they have followed the protocols of their Center and Local regulations on the publication of patient data.

Rights to privacy and informed consent: The authors have obtained the informed consent of the patients and/or subjects referred to in the article. This document is in the possession of the correspondence author.

Conflicts of Interest

Authors declare no conflict of interest regarding the present study.

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