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# Immunoexpression of E-cadherin, Snail and Twist in colonic adenocarcinomas

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**Abstract**

Epithelial–mesenchymal transition (EMT) is an important mechanism in tumor progression. Snail is a transcription factor, expressed in cells which have undergone almost complete EMT and have left the tumor, and Twist is considered important in the process of metastasis, both playing a major role in EMT by indirect inhibition of E-cadherin. The study analyzed the immunoexpression of E-cadherin, Snail and Twist in 46 cases of colonic carcinomas in comparison with some histopathological prognostic factors. The quantification of reactions was done by using a composite score (CS) resulted from multiplying the percentage of marked cells with the intensity of immunostaining. The majority of cases were moderately differentiated tumors, corresponded to stage III, with vascular and perineural invasion. All cases presented positive cytoplasmic and nuclear signals for Snail and Twist. The immunostaining for both markers was intense, with the highest values of CS in G2 and G3 advanced, invasive vascular colonic carcinomas, in comparison with G1, early stage lesions. We found positive significant linear correlation of Snail and Twist expression. The results obtained indicate the implication of Snail and Twist in colonic carcinoma aggressiveness, useful aspect in the oncological evaluation of patients and guided therapy.

***Keywords***: CRC, E-cadherin, Snail, Twist, tumor stage, tumor grading.

## Introduction

Colorectal carcinoma (CRC) is the third most frequent type of cancer worldwide [1]. Epithelial–mesenchymal transition (EMT) refers to the process by which epithelial cells release from parental tissue, losing their polarity and are converted to mesenchymal phenotypes, for example cells of the primary tumor at the site of invasion, which have suffered phenotypical conversion, invade and metastasize [2]. Invasion represents the first step in the cascade of events, which lead to metastasis and EMT is considered one of the central mechanisms, which induces this process [3, 4].

In EMT regulation, several signaling pathways are implicated, primary role in this process being represented by Snail and Twist transcription proteins, associated with invasion, metastasis and with poor prognostic in patients with CRC [2–4]. The primary mechanism by which EMT is induced is represented by E-cadherin inhibition, by the binding of Snail and Twist to the E-boxes situated in E-cadherin promoters [5, 6].

The present study followed the correlation of Snail and Twist with some histopathological (HP) parameters with known prognostic value in CRC.

## Materials and Methods

The study included 46 cases of colonic carcinomas obtained from the Clinics of Surgery of Emergency County Hospital of Craiova, Romania, during 2017 and diagnosed in the Laboratory of Pathology of the same Hospital. Biological material consisted of specimens of colectomy and hemi-colectomy, fixed in 10% buffered formalin and then processed by the classical HP technique consisting on paraffin embedding and Hematoxylin–Eosin (HE) staining. Tumor staging was made in concordance with the *World Health Organization* (WHO) 2010 Guidelines [7]. HP parameters (grading, tumor staging, vascular and neural invasion) were analyzed in comparison with immunoexpression of rabbit–anti-human monoclonal E-cadherin (36B5) and rabbit–anti-human polyclonal antibodies, Snail (NBP1-822) and Twist (ab50581), immunoglobulin G (IgG) isotype. For the immuno-histochemical (IHC) analysis, serial sections were used, which were processed by Labeled Streptavidin–Biotin (LSAB)+ System–Horseradish peroxidase (HRP) (Dako, Redox, Romania, code K0675), signal developing being made with the 3,3'-Diamino-benzidine (DAB) tetrahydro-chloride chromogen (Dako, Redox, Romania, code K3468). We used E-cadherin (ready-to-use), 1:100 dilution for Snail, 1:500 dilution for Twist, and antigenic pretreatment was done with microwaving in citrate buffer pH 6.

The quantification of IHC reactions was realized by using a composite score (CS) resulted by multiplying the percentage of marked cells with the immunostaing intensity. Depending on the number of marked tumoral cells, cases were divided into several categories, respectively 0 (absence of marked cells), 1 (less then 1% marked cells), 2 (10–25% marked cells), 3 (25–50% marked cells), 4 (more then 50% marked cells). The immunostaing intensity was divided into four categories, respectively 0 (absent), 1 (weak), 2 (moderate) and 3 (intense). The images were

acquired with the Nikon Eclipse E600 microscope and imaging software Lucia 5.

Statistical analysis was made by $\chi^2$ (*chi*-square) test and Pearson's comparison tests, within *Statistical Package for the Social Sciences* (SPSS) 10 software. Mean values were reported as standard deviation (SD). The results were considered significant for values of $p<0.05$. For the statistical analysis, the resulted scores were considered low for values between 1 and 4 and high for values between 6 and 12.

The study was approved by the Local Ethics Committee (No. 42/27.03.2018).

## Results

The analysis of the 46 cases indicated that most were moderately differentiated tumors (G2 – 45.7%) and corresponded to stage III (32.6%), 69.6% presented vascular invasion and 39.1% perineural invasion (Table 1).

**Table 1 –** ***Relation between histopathological parameters and E-cadherin, Snail and Twist immunoexpression***

| Parameters | Variables and No. of cases (%) | E-cadherin | | Snail | | Twist | |
|---|---|---|---|---|---|---|---|
| | | *%* | *Score* | *%* | *Score* | *%* | *Score* |
| *Tumoral stage* | I – 6 (13%) | 95.83±4.91 | 10 | 31.66±11.25 | 7.3 | 33.33±11.69 | 3.83 |
| | II – 13 (28.3%) | 84.28±20.92 | 8.14 | 70.76±20.7 | 11.15 | 77.81±17.7 | 9.68 |
| | III – 15 (32.6%) | 73.07±15.61 | 7.23 | 79±11.83 | 11.8 | 82.69±10.72 | 10.53 |
| | IV – 12 (26.1%) | 58.25±25.04 | 4.76 | 90.83±9.25 | 11.33 | 93.81±8.73 | 11.27 |
| | | $p$=0.006 | | $p$=0.029 | | $p$<0.001 | |
| *Grading* | G1 – 9 (19.6%) | 89.37±20.6 | 8.75 | 42.22±20.63 | 8 | 45.55±22.28 | 5.77 |
| | G2 – 21 (45.7%) | 80.08±19.07 | 7.96 | 80.23±13.64 | 11.66 | 82.85±12.99 | 10.71 |
| | G3 – 16 (34.8%) | 61.07±24.5 | 5.92 | 82.5±18.52 | 11.56 | 87.18±15.7 | 10.12 |
| | | $p$=0.028 | | $p$=0.001 | | $p$<0.001 | |
| *Vascular invasion* | Present – 32 (69.6%) | 70.53±24.12 | 7.42 | 82.34±14.7 | 11.46 | 87.34±11.07 | 10.53 |
| | Absent – 14 (30.4%) | 84.47±19.05 | 7.68 | 53.57±25.67 | 9.64 | 53.57±23.89 | 7.28 |
| | | $p$=0.908 | | $p$=0.007 | | $p$<0.001 | |
| *Perineural invasion* | Present – 18 (39.1%) | 71.36±23.56 | 6.48 | 89.44±10.83 | 11.55 | 88.88±11.57 | 11.11 |
| | Absent – 28 (60.9%) | 86.15±18.75 | 10.15 | 63.39±22.69 | 10.5 | 69.46±24.27 | 8.53 |
| | | $p$=0.828 | | $p$=0.15 | | $p$=0.035 | |

G1: Well differentiated CRC, G2: Moderately differentiated CRC, G3: Low differentiated CRC; CRC: Colorectal carcinoma.

E-cadherin reaction was identified with a membranar and cytoplasmic pattern in 75.76% of cases (Figure 1, A and B). E-cadherin intensity and immunostained cell percentage were different depending on grading. Well-differentiated carcinomas (G1) had an intense reaction, with a mean CS value of 8.75, moderately differentiated cancers (G2) had an intense reaction with a mean CS value of 7.96, while low differentiated cancers (G3) had a low reaction with a mean CS value of 5.92, aspect which is statistically insignificant (Figure 1C).

Depending on tumor stage, we observed a decreased with advancement of tumor stage, respectively 10 (stage I), 8.14 (stage II), 7.23 (stage III) and 4.76 (stage IV), which was statistically significant (Figure 1D).

Related to vascular invasion, we observed an increased CS mean values in non-invasive colonic carcinomas compared with invasive ones (7.68 *vs.* 7.42), without a significant statistical aspect ($p$=0.908, *chi*-square test). Similarly, the analysis of the relation between CS and perineural invasion indicated an increase in the presence of perineural invasion (6.48 *vs.* 10.15) but without a significant statistical aspect ($p$=0.828, *chi*-square test).

Snail immunostaining was identified with a nuclear and cytoplasmic pattern in 73.58% of cases (Figure 2, A and B). Snail intensity and the percentage of immunostained cells were different depending on grading. Well-differentiated carcinomas (G1) indicated an intense reaction with a mean CS of 8. Also, moderately differentiated (G2) and low differentiated carcinomas have shown a high intensity with a mean CS value of 11.66 (G2), respectively 11.56 (G3), aspect which as statistically significant (Figure 2C).

Depending on tumor stage, we observed an increase of mean CS value in advanced lesions, respectively 7.3 (stage I), 11.15 (stage II), 11.8 (stage III) and 11.33 (stage IV), aspect which was statistically significant (Figure 2D).

Related to vascular invasion, we observed an increased CS mean values in invasive colonic carcinomas compared with non-invasive ones (11.46 *vs.* 9.64), aspect which was statistically significant. Similarly, the analysis of the relation between CS and perineural invasion indicated an increase in the presence of perineural invasion (11.55 *vs.* 10.5) but without a significant statistical aspect ($p$=0.15, *chi*-square test).

Twist immunostaining was identified with a nuclear and cytoplasmic pattern in 77.06 of cases (Figure 3, A and B). Twist intensity and the percentage of immunostained cells were different depending on grading. Well-differentiated carcinomas (G1) indicated a high CS with a mean value of 5.77. Also, moderately differentiated (G2) and low differentiated carcinomas have shown a high intensity with a mean CS value of 10.71 (G2), respectively 10.12 (G3), aspect which was statistically significant (Figure 3C).

Depending on tumor stage, we observed an increase of mean CS value in advanced lesions, 3.83 (stage I), 9.68 (stage II), 10.63 (stage III) and 11.27 (stage IV), aspect which was statistically significant (Figure 3D).

Related to vascular invasion, we observed an increased CS mean values in invasive colonic carcinomas compared with non-invasive ones (10.53 *vs.* 7.28), aspect which was statistically significant. Similarly, the analysis of the relation between CS and perineural invasion, indicated an increase in the presence of perineural invasion (11.11 *vs.* 8.53). The relation between Twist immunostaining and HP factors was statistically significant ($p<0.05$, *chi*-square test).

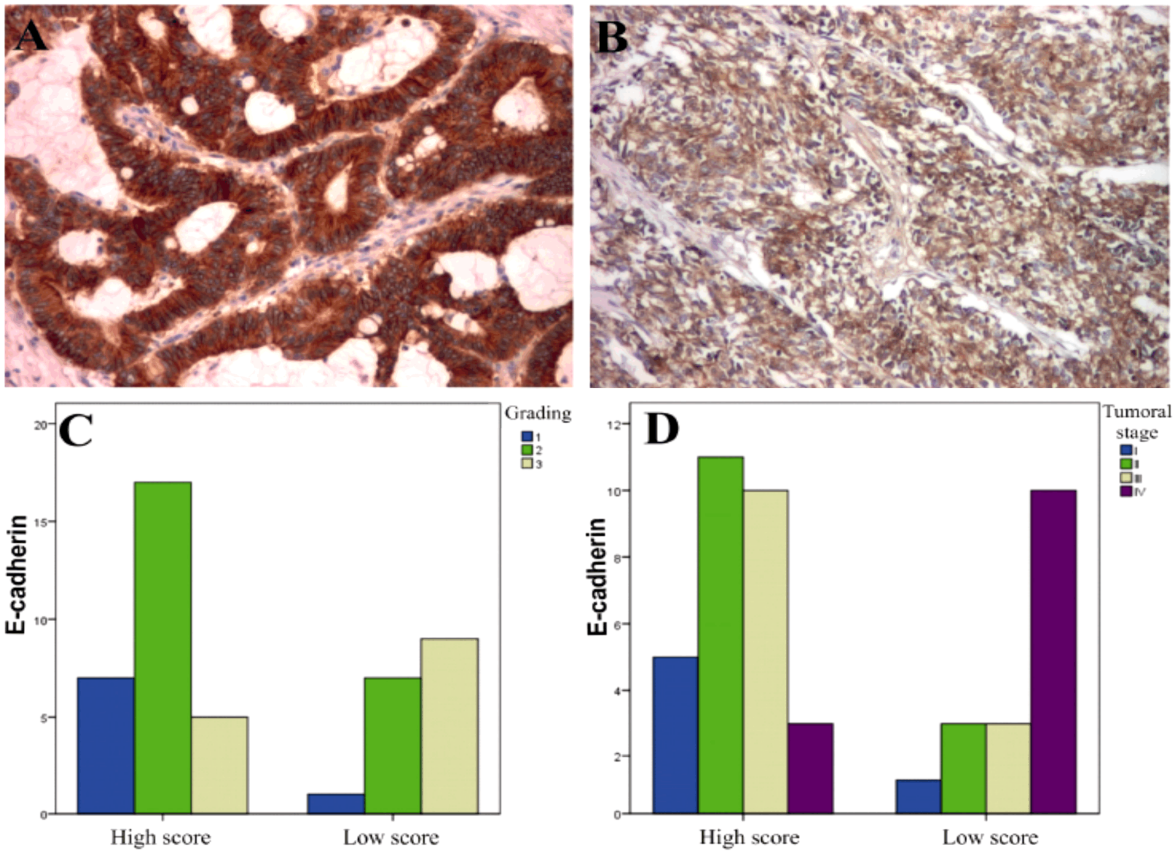


**Figure 1** – ***(A) Well-differentiated colonic carcinoma; (B) Low differentiated colonic carcinoma; (C) Cases distribution depending on E-cadherin scores and grading; (D) Cases distribution depending on E-cadherin scores and tumoral stage. Anti-E-cadherin antibody immunomarking: (A and B) ×100.***

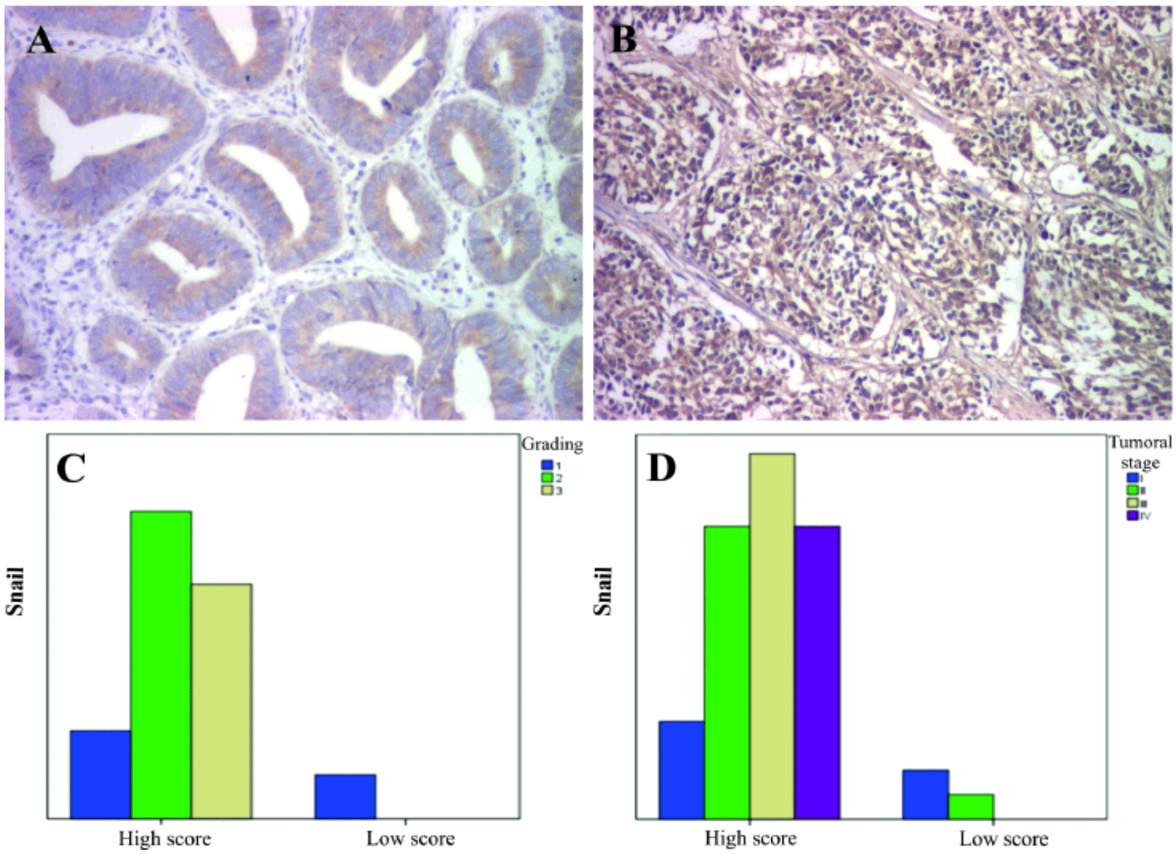


**Figure 2** – ***(A) Well-differentiated colonic carcinoma; (B) Low differentiated colonic carcinoma; (C) Cases distribution depending on Snail scores and grading; (D) Cases distribution depending on Snail scores and tumoral stage. Anti-Snail antibody immunomarking: (A and B) ×100.***

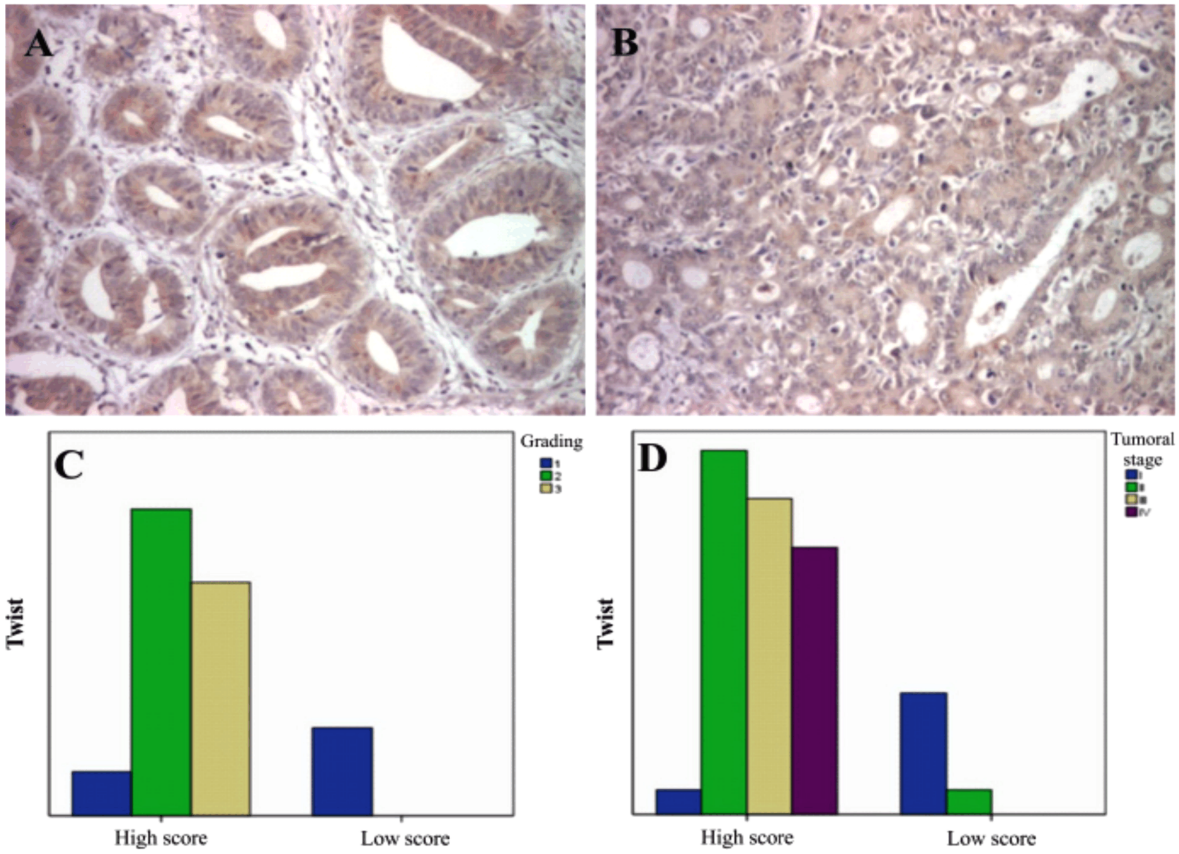


**Figure 3** – ***(A) Well-differentiated CRC; (B) Low differentiated colonic carcinoma; (C) Cases distribution depending on Twist scores and grading; (D) Cases distribution depending on Twist scores and tumoral stage. Anti-Twist antibody immunomarking: (A and B) ×100. CRC: Colorectal carcinoma.***

Statistical analysis showed positive significant linear correlation of Snail and Twist ($p$<0.05, Pearson's test) (Figure 4).

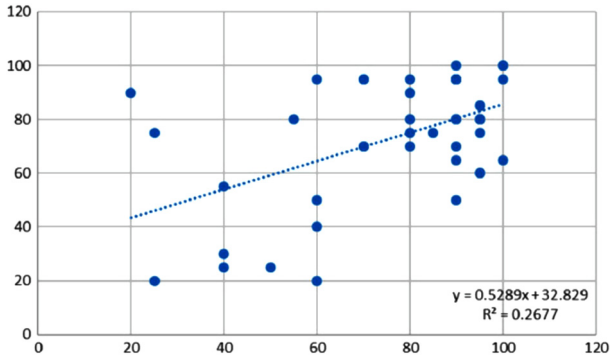


**Figure 4** – ***Twist and Snail percentage values distribution.***

## Discussions

CRC is the third most diagnosed type of cancer in men but also in women. Tumor staging represents the most important prognostic factor, for patients with CRC, being associated with low grading, vascular and perineural invasion [8].

Most of the analyzed cases were moderately differentiated tumors, corresponding to stage III and frequently presented vascular and perineural invasion, with results statistically significant between the parameters used. Acquired data are in concordance with those found in literature, in conformity with other studies on different HP models. Fujii *et al.* and Hashmi *et al.* demonstrated in separate publications that the majority of tumors are diagnosed in late stages of the disease III/IV, being associated with vascular and perineural invasion [9, 10]. Also, grading changes in parallel with the increase of tumoral staging, most frequent tumors being moderately differentiated, aspect that was also demonstrated by Barresi *et al.* [11].

EMT is considered one of the essential mechanisms of aggressive carcinomas. Aberrant activation of EMT refers to the process by which epithelial tumor cells lose their polarity and are converted to mesenchymal phenotype, thus acquiring migrational ability. This process is accompanied by different modifications, such as epithelial phenotype inhibition (E-cadherin inhibition) and evidentiation of mesenchymal markers, but also the modification of the interaction between related proteins, such as Snail and Twist [12]. The definitory role of EMT is represented by the direct link to invasion, metastasis, and unfavorable prognostic of patients with CRC [13, 14]. In different types of carcinomas, transcriptional proteins Snail and Twist can promote EMT independently or by synergistically mechanisms [12].

E-cadherin is expressed in most epithelial tissues and forms a tight junction, which connects adjacent cells. Loss of E-cadherin leads to loss of cell adhesion, tumor progression, metastasis and an unfavorable prognostic in different cancers [15, 16].

E-cadherin expression analysis showed its presence in 75.76% of studied cases in membrane and cytoplasm. Similar to our study, another study found in literature

showed E-cadherin expression in the membrane and cytoplasm [17]. Elzagheid *et al.* concluded that the odds of a recurrent disease are influenced by the presence of aberrant cytoplasmic expression of E-cadherin, which would be of clinical and therapeutic interest [18]. E-cadherin markings were intense, with the highest values of mean CS in colonic carcinomas G1 (8.75) and G2 (7.96), in comparison with G3 (5.92) being statistically significant. Another study evidenced E-cadherin membranary and cytoplasmic immunoreaction and the association with tumor differentiation. Thus, they concluded that well and moderately differentiated tumors have a higher E-cadherin expression in comparison with low differentiated, the association being statistically significant [19].

In our study the tumor cells intensity decreased in advanced tumor stage, aspect which was statistically significant. Data found in literature, has shown that low expression of E-cadherin was significantly correlated with tumor differentiation and advanced tumor stage [20]. Association of E-cadherin immunoreaction with perineural and vascular invasion was statistically insignificant, but many studies demonstrated the opposite [21, 22].

Snail is considered having a prognostic role in CRC and is an EMT promoter, which mediates invasiveness and metastasis in many types of malignant tumors [23, 24].

Snail expression analysis in colonic carcinomas indicated positivity in 74% of cases, which presented overexpression of this protein, with a cytoplasmic and nuclear pattern. Similarly, Fan *et al.* demonstrated that 78% of analyzed cases presented Snail overexpression [6]. Yang *et al.* showed that Snail overexpression and from this EMT correlates with aggressive CRC [16]. In literature, many other studies showed Snail overexpression in CRC [25, 26]. Luo *et al.* confirmed the presence of Snail expression in the cytoplasm (37.7%) and in the nucleus (49.2%) of tumoral cells of nasopharyngeal cancers in a study of 122 cases of carcinomas [27]. On the other hand, some publications quantify nuclear Snail immunostaining in tumoral cells and is considered a poor prognostic factor [28, 29], without evidence of cytoplasmic staining. Snail immunostaining was intense with the highest values of mean CS in CRC G2 (11.66) and G3 (11.56), aspect which was statistically significant, data similar with those found in literature [30].

Also, we identified significant association between Snail immunostaining and vascular invasion but we have not found a statistically significant association with perineural invasion. In literature, it is demonstrated the association of Snail overexpression with perineural and vascular invasion, aspect which suggests the ability to invade and migrate of this marker and a poor prognostic [30–32].

Twist overexpression was observed not only in CRC but also in other carcinomas, such as breast, prostate, lung, superior gastrointestinal tract, being associated with tumor progression and early metastasis [33].

We observed that Twist immunostaining was intense with nuclear and cytoplasmic pattern in 77.06% of cases and superior in colonic carcinomas G3. Comparatively, Mohammed *et al.*, in a study which composed of 49 cases of CRC, demonstrated that 42 of the cases presented positive Twist expression in the cytoplasm and 29 positive Twist expression in the nucleus being significantly associated with low differentiated tumors, advanced disease stage and which a have a tendency to manifest vascular invasion [13]. Valdés-Mora *et al.* concluded that Twist overexpression in CRC is associated with tumor progression, invasion and metastasis [5]. Similar data is found in other publications, nuclear and cytoplasmic staining being present not only in CRC but also in other types of carcinomas, such as gastric and hepatocellular [33]. Twist association with advanced stages of disease was long studied, fact which is seen by the numerous statistical data in literature [34, 35].

In our study, the relation between Twist vascular and perineural invasion was statistically significant. Zhu *et al.* showed that Twist is in a close relation with invasion, tumor grading, lymph node metastasis and with advanced tumor stages [36]. Similarly, Yang *et al.* suggested that Twist expression is essential in the process of metastasis; immunostaining of this protein revealed to be a marker of aggressive CRC and can lead to metastasis. In this case, tumors that show Twist overexpression show a much more aggressive behavior and begin the metastatic process even though secondary tumors are not visible macroscopic [37]. Several studies indicated that Twist is a trustworthy marker of CRC aggressiveness, by showing the progression of metastasis and prognostic [34, 36].

Furthermore, we concluded significant positive linear correlation of Snail and Twist expression ($p<0.05$, Pearson's test) an aspect which suggests that the expression of the two EMT markers was associated with the presence of a minimal survival rate, similar to data found in literature [12].

## Conclusions

In this study, E-cadherin reaction decreased in advanced tumoral stages, while Snail and Twist immunoexpression was significantly superior in high grade invasive CRC and in advanced stages. This aspect suggests the implication of these markers in colonic carcinomas EMT, and the obtained results support the utility of these proteins in the stratification of patients, with a possible prognostic and therapeutic role.

### Conflict of interests

The authors declare that they have no conflict of interests.

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