**Rezumat**

Expresia imunohistochimică aberantă a markerelor EMT, Vimentina și E-caderină și OCT3/4 este corelată cu parametri histopatologici adverși în adenocarcinomul colorectal.

**Scope:** Deși managementul clinic al cancerului colorectal a fost îmbunătățit semnificativ, acesta se confruntă cu o incidență în creștere în rândul tinerilor și în rândul populațiilor din țările în curs de dezvoltare. Mai mult decât atât, diagnosticul este pus mai ales în stadii avansate, când resursele terapeutice sunt limitate. Prin urmare, avem nevoie de noi biomarkeri pentru diagnosticare și noi ținte terapeutice. Evenimentul cheie care duce la invazie și metastază este tranziția epitelial-mezenchimală (EMT), care poate fi studiată cu markeri IHC. Am urmărit corelarea expresiei markerelor legați de EMT (Vimentină și E-caderină) și a unui marker de celule stem (OCT 3/4) cu parametrii clinicopatologici ai tumorilor.

**Material și metode:** Au fost evaluate probe de rezecție chirurgicală de la 30 de pacienți cu cancer de colon fără tratament neo-adjuvant, internați în perioada 2018-2021. Au fost efectuate teste imunohistochimice pentru a investiga expresia markerelor legați de EMT și OCT 3/4 în celulele tumorale.
**Abstract**

*Background:* Although clinical management for colorectal cancer has been markedly improved, it is faced with a growing incidence among the young and among those in developing nations. Furthermore, diagnosis occurs mostly in advanced stages, when the therapeutic resources are limited. Therefore, we need new biomarkers for diagnostics and therapeutic targets. The key event that leads to invasion and metastasis is the epithelial to mesenchymal transition (EMT), which can be studied with IHC markers. We aimed to correlate the expression of EMT-related markers (Vimentin and E-cadherin) and a stem cell marker (OCT3/4) with the clinicopathological parameters of the tumors.

*Material and Methods:* Surgical resection specimens from 30 treatment-naive colon cancer patients, hospitalized from 2018 to 2021 were assessed. Immunohistochemical tests were performed to investigate the expression of EMT-related markers and OCT3/4 in tumor cells.

*Results:* Vimentin, OCT3/4 positivity and loss of E-cadherin were significantly associated with tumor grade, tumor budding, invasive tumor front, and lymph node metastasis.

*Conclusions:* Vimentin, E-cadherin and OCT3/4 might serve as a panel of biomarkers that can aid in the prognostication of patients, with the added potential of being oncotargets.

*Key words:* colon adenocarcinoma, EMT, Vimentin, E-cadherin, OCT3/4, tumor budding, invasive tumor front
as spindle-like morphology that lacks vertical polarity, causing increased motility and invasiveness of the cancer cells (4-5). The epitome of the EMT process is the up-regulation of mesenchymal markers and down-regulation of structural adhesion proteins. During the EMT the tumor cells start to lose the expression of E-cadherin, an event that leads to the reduction of the cell-to-cell adhesion. This is paralleled by the elevated expression levels of the mesenchymal marker Vimentin (6-8). Besides serving as a functional and morphological framework for tissue invasion and metastasis, the EMT is also an obstacle in the success of cancer therapy by being a pivotal process in tumor heterogeneity and therapy resistance (9-10).

In COAD there seems to exist a morphological indicator of undergoing EMT, in the form of tumor buds (12), which are defined as the presence of single tumor cells or small tumor clusters composed of up to four cells at the invasive front of the tumor and it has been incorporated as an additional prognostic factor for COAD in the 8th Edition of the TNM Classification of Malignant Tumors (13) and the WHO classification of 2019 (15). It has been showed that tumor buds are at least partially synonymous with EMT, as they lose E-cadherin expression and co-express cytokeratin and Vimentin (14).

Vimentin is a 57 KD, type III intermediate filament protein that is ubiquitously expressed in normal mesenchymal tissues, in various stages of development, contributing to cellular and tissular structure and integrity (11) and it is strongly upregulated following injury to various tissues (16). VIM influences the cell shape and mobility during the EMT, being abnormally expressed in epithelial cells. Furthermore, gene knockdown of VIM resulted in decreases in cell mobility and invasiveness in colon carcinoma cell lines (17). Thus, it can be used to identify cancer cells undertaking EMT as there is a positive association of Vimentin expression with augmented invasiveness and metastasis (6,19).

E-cadherin (E-CAD) is a calcium-dependent transmembrane glycoprotein, encoded by the CDH1 gene, and a member of the “classical” or type I Cadherins subfamily (18). Although it does not possess enzymatic activity, it acts as a tumor suppressor in numerous human epithelial tumors, inhibiting migration and metastasis (20-21). E-CAD facilitates the assembly of intercellular junctions and it serves as an adhesion molecule. It is localized at the basolateral surface of epithelial cells, and it is involved in cell-to-cell interactions, both in normal and cancer cells (22). Unlike Vimentin, studies have shown that inhibition of CDH1 gene expression in colon cancer cell lines promotes cell migration and invasion (23-24), and it can be used to identify cells that undergo EMT by showing a negative correlation (i.e., loss of expression). Besides structural proteins like VIM and E-CAD, several other EMT associated transcription factors like Slug, Twist, Zeb1 and Zeb2 have been reported as markers for aggressive tumor markers in various cancers including colorectal carcinoma (25-26).

Recently, the octamer-binding transcription factor 4 (also known as OCT3/4, OTF3, or POU5F1), a transcription factor that ensures survival and maintains pluripotentiality of embryonic stem cells (27), has been showed to be involved in the progression of various types of cancer, including breast, non-small cell lung carcinoma, gliomas, gastric cancers, and COAD (28-34). Being a stem cell marker, it is currently widely used for the diagnosis of germ cell tumors (36). One previous study demonstrated that OCT4 gene knockdown induced EMT-like cell morphological changes and EMT marker expression alteration, indicating that OCT4 is involved in the EMT process in colorectal cancer cells (35).

Therefore, this study was performed in part to evaluate the expression of EMT markers, Vimentin and E-cadherin and the correlation with the clinicopathological parameters in COAD matched with normal tissue. Our secondary objective was to evaluate the expression of OCT3/4 in comparison with the EMT markers and the clinicopathological parameters in hoping to better understand the relationship between stem cells and EMT.
Material and Methods

Our tumor series comprised 30 colorectal carcinoma cancer patients, who underwent surgical resection, between 2018 and 2021. Inclusion criteria: patients with colorectal cancer confirmed on resection specimens, operated between 2018-2021, aged >18 years, with complete clinical and paraclinical data, no neoadjuvant treatments prior to surgical resection, with complete immunohistochemical results for Vimentin, E-cadherin, and OCT3/4. Patients with unresected colorectal cancers, those with chemotherapeutic treatments prior to colonic resection, those with incomplete clinical-paraclinical data, or those in whom immunohistochemical testing was not performed were excluded.

All the relevant clinicopathologic characteristics obtained from staging (sex, age, tumor localization, tumor stage, tumor grade, histological type, tumor budding, number of involved lymph nodes, presence of satellite nodules, lymph node stage, type of tumor front, venous-lymphatic invasion and perineural invasion) were assessed for all patients.

All tumors were chemo-/radiation-naïve, none of the patients received chemo-radiotherapy prior to surgery. Surgical specimens were histologically evaluated based on the 8th edition of the American Joint Committee on Cancer guidelines. Hematoxylin-Eosin (HE) stained sections were reviewed by two pathologists, who confirmed the diagnosis of COAD and staged the tumors.

Tumor Budding Evaluation

The evaluation of tumor buds was done with regards to the criteria established by the International Tumor Budding Consensus Conference (ITBCC) (36). Hotspot areas were identified at the invasive front by light microscopy on histology slides stained with the usual, HE stain, using a scanning magnification. Tumor buds were defined as single tumor cells or small clusters composed of maximum 4 tumor cells. The buds were counted on a single field of a 20 x objective lens, corresponding to a 0.785 mm² area. The cases were classified based upon the number of buds in order of severity into Bd1 –low (0–4 buds/0.785 mm²), Bd2 – intermediate (5–9 buds), and Bd3 – high (≥10 buds).

Lymph Node Status Evaluation

Based on the total number of metastatic lymph nodes, the cases were further divided into N groups as follows: a) pN0 (no metastatic lymph nodes); b) pN1 (metastases in one or two lymph nodes); and c) pN2 (more than two metastatic lymph nodes). We also calculated the metastatic lymph node index by dividing the number of metastatic lymph nodes to the total number of lymph nodes identified in the surgical specimens.

Type of Tumor Front Evaluation

Tumor growth pattern at the tumor invasion front was assessed at scanning magnification and categorized as expansive or infiltrative as described by a previous study (37). The growth pattern was considered expansive when the tumor margin was non-infiltrative, pushing and well-circumscribed. It was considered infiltrative when tumor glands or irregular trabeculae or nests of tumor cells invaded in a haphazard manner with widespread infiltration of adjacent tissue without a clear, pushing border.

Venous Invasion Type

Venous invasion, when present, was divided into two groups, namely extramural type (EMVI - beyond muscularis propria) or intramural type (IMVI - restricted to submucosa or muscularis propria). This distinction was performed because it has been showed by multivariate analysis that EMVI is an independent adverse prognostic factor for COAD and is correlated with liver metastasis (38).
**Immunohistochemistry (IHC)**

IHC tests were performed on 2 µm sections from formalin-fixed paraffin-embedded tissues (fixed in 10% neutral buffered formalin), from each tumor, by utilizing the CONFIRM anti-Vimentin (V9) primary antibody (Ventana Medical Systems, USA). An automated immunostainer (Ventana Bench Mark XT) was used to perform the IHC procedure. For E-cadherin and OCT3/4 we used the 36B5 and N1NK antibody clones respectively (Leica Microsystems GmbH, Germany). An automated immunostainer (BOND-III Fully Automated IHC and ISH Staining System - Leica Microsystems GmbH, Germany) was also used for these antibodies. We anticipated the risk of false negative reactions due to heterogenous staining by assessing tissue microarrays, so we only used full slides. The Vimentin IHC slides were evaluated by examining cytoplasmic staining. Vimentin cytoplasmic expression in more than 5% of tumor cells was considered positive and less than 5% of tumor cells was considered negative (39). For E-cadherin, membranous staining was considered positive and a total loss of expression in more than 5% of the tumor cells, or a partial loss in more than 20% was considered significant. For OCT3/4 any convincing cytoplasmic or nuclear staining in more than 1% of the tumor cells was considered positive.

**Statistical Analysis**

The data were processed using SPSS version 23.0, running on a Windows 10 operating system. For descriptive statistics, the means and standard deviations were calculated, the medians and quartiles for the quantitative variables, and the frequency and percentage for qualitative variables, respectively.

Compared to the quantitative data, depending on the normality of the data, the Student t-test (Independent Sample T-test) (for two groups with normally distributed data) and Mann-Whitney (for data that do not have a normal distribution) were used, respectively.

Quantitative data were tested for normality (Shapiro-Wilk test) and variant homogeneity using the Levene test. Fisher's Exact Test was used for the data. The probability of error less than 5% (p <0.05) was considered the threshold of statistical significance.

**Results**

**Clinicopathological parameters**

A total of 30 cases of colorectal cancers were analyzed. Of the 30 cases, 11 (37%) were male, and 19 female (63%). The age range was between 47 and 87 years, with a median of 67.5 years. The right side was involved in 18 cases (60%), the left being involved in 12 cases (40%). Histologically, 27 cases were conventional adenocarcinomas, and 3 cases were mucinous adenocarcinomas, 90%, and 10% respectively. Three tumors were well differentiated, 23 were moderately differentiated, and 4 were poorly differentiated (10%, 76% and 14% respectively). Regarding stage, 2 tumors were T2, 18 were T3 tumors and the remaining 10 being stage 4 (7%, 60% and 33% respectively). Of the 30 cases, lymphovascular invasion was found in 28 cases (93%), with 17 of the EMVI type (57%), the remaining 11 being of the IMVI type (36%). Tumor budding was mostly evaluated as Bd1, consisting of 18 cases (60%), while Bd2 was encountered in 7 cases (23%) and Bd3 in 5 (17%) cases. Perineural invasion was found in 17 cases (57%) the rest of 13 cases no showing PNI (43%). The tumor front was infiltrative in 11 cases and pushing in 19 cases (37% vs 63%). The mean number of lymph nodes harvested was 28.46, and the average number of lymph nodes with metastases was 2.5: 16 patients had a lymph node ratio of zero and 14 patients more than zero (Table 1).

Of the 30 cases, 11 showed abnormal cytoplasmic Vimentin positivity (37%), (Fig. 1). E-Cadherin loss of expression found in 17 cases (Fig. 2). Only 7 cases showed OCT3/4 positivity (Fig. 3, Table 1).
Table 1. Demographic and clinicopathological characteristics of patients

|                          | Patient numbers |
|--------------------------|-----------------|
| **Sex**                  |                 |
| M                        | 11              |
| F                        | 19              |
| **Age**                  |                 |
| >70                      | 14              |
| ≤ 70                     | 16              |
| **Side**                 |                 |
| Left                     | 12              |
| Right                    | 18              |
| **Histological type**    |                 |
| Conventional             | 27              |
| Mucinous                 | 3               |
| **Grading**              |                 |
| G1                       | 3               |
| G2                       | 23              |
| G3                       | 4               |
| **T stage**              |                 |
| 2                        | 2               |
| 3                        | 18              |
| 4                        | 10              |
| **N Stage**              |                 |
| N0                       | 15              |
| N1                       | 6               |
| N2                       | 9               |
| **Tumor front**          |                 |
| Infiltrative             | 11              |
| Pushing                  | 19              |
| **Tumor budding**        |                 |
| Bd1                      | 18              |
| Bd2                      | 7               |
| Bd3                      | 5               |
| **Venous invasion**      |                 |
| Not identified           | 2               |
| EMVI                     | 17              |
| IMVI                     | 11              |
| **Perineural Invasion**  |                 |
| Yes                      | 17              |
| No                       | 13              |
| **Lymph node ratio**     |                 |
| >0                       | 14              |
| =0                       | 16              |
| **Vimentin**             |                 |
| Positive                 | 11              |
| Negative                 | 19              |
| **E-cadherin**           |                 |
| No loss of expression    | 13              |
| Loss of expression       | 17              |
| **OCT3/4**               |                 |
| Positive                 | 7               |
| Negative                 | 23              |

Statistical analysis of Vimentin, E-cadherin and OCT 3/4 expression in relation to clinicopathological parameters

For evaluating the correlation of Vimentin, E-cadherin and OCT3/4 expression with sex, age, sideness, pT category, pN category, number of metastatic lymph nodes, histological grade, type of tumor front, tumor buds, lymphovascular and perineural invasion were used for statistical tests. We found no significant statistical correlation between the expression of Vimentin, E-cadherin or OCT3/4 and the age, sex, location of the tumors or histological type. Vimentin expression was correlated with tumor grade, being significantly more expressed in G3 tumors (p_value=0.003768). There was a significant correlation between Vimentin positivity and the degree of tumor budding, with an increased positivity in cases with Bd-2 and Bd-3 (p_value= 0.000009). Although there was no correlation with the presence of lymphovascular invasion (p_value=0.519540), there was a significant correlation between the positivity of Vimentin and the type of vascular invasion, being more
frequent in the EMVI type (p_value=0.007857). There were also statistical correlations between Vimentin positivity and pT and pN categories, as it tended to be associated with higher categories (p_value=0.001360, and 0.000114, respectively). Cases with perineural invasion also tended to present Vimentin positivity (p_value=0.000317). There was a strong association of Vimentin expression with the invasive tumor front (p_value=0.000004) (Table 2).

Loss of expression of E-cadherin was increased in G3 tumors compared to G1 and G2 (P value=0.005902). Cases with low tumor budding (Bd-1) showed significantly lower loss of expression compared to cases with high tumor budding (Bd-3) (p_value=0.000267). There was no correlation with pT categories, but we found correlations between loss of E-CAD and high pN categories (p_value=0.039094) and increased number of metastatic lymph nodes (p_value=0.015858). Unlike Vimentin, there was no correlation of E-CAD expression and perineural invasion or lymphovascular invasion (p_value=0.071120 and 0.491954, respectively), however, there was a strong correlation between loss of E-CAD expression and an invasive tumor front (p_value=0.022746) (Table 2).

Besides the previously mentioned, OCT3/4 positivity did not show correlations with perineural invasion, lymphovascular invasion, pT and pN categories. We did, however, found correlations with high tumor grade (p_value=0.032647), high tumor budding (p_value=0.003195), number of
metastatic lymph nodes (p_value=0.011315), and invasive tumor front (p_value=0.004474). Lastly, OCT3/4 positivity was not correlated with Vimentin expression, but it did show correlation with loss of E-CAD (p_value=0.024725) (Table 2).

Discussions

Invasion and metastasis are the most dangerous characteristics of malignant tumors. The abnormal expression of cell adhesion molecules plays an important role in improving the mobility of tumor cells which leads to enhanced migration properties and to the formation of metastasis. During EMT, the epithelial cancer cells undergo transformation and they develop mesenchymal properties, losing E-cadherin (40) and gaining Vimentin expression. In this study, we investigated a heterogeneous (with regard to site, tumor budding, stage, tumor grade, invasion front, lymphovascular invasion, perineural invasion) cohort of colon cancer patients for expression of Vimentin, E-cadherin and the stem cell marker OCT3/4. Our study shows that expression of Vimentin in tumors correlates with tumor

| Table 2. | Vimentin expression and correlation with clinicopathological parameters |
|----------|------------------------------------------------------------------------|
|          | VIM= neg (N=19)             | VIM= pos (N=11)             | p_value (test)     |
| Age      | 67.63±11.3930              | 67.27±10.8911              | 0.933303 (Independent Samples T Test) |
| Sex      | 11/19 (57.9%)              | 8/11 (72.7%)               | 0.466146 (Fisher’s Exact Test) |
| Histology| Conventional               | Mucinous                   | 0.279310 (Fisher’s Exact Test) |
| Tumor grade | G1 (15.8%)                | 0/11 (0%)                  | 0.003768 (Likelihood Ratio) |
| Tumour Budding | Bb-1 (89.5%)         | 1/11 (9.1%)                | 0.000009 (Likelihood Ratio) |
| Lymphovascular invasion | 0/11 (9.1%)            | 0/11 (0%)                  | 0.519540 (Fisher’s Exact Test) |
| N category | 0 (73.7%)                  | 1/11 (9.1%)                | 0.000114 (Likelihood Ratio) |
| T category | 2 (10.5%)                  | 0/11 (0%)                  | 0.001360 (Likelihood Ratio) |
| Number of positive lymphnodes | 4.00 (2.00, 9.00)       | 4.00 (2.00, 9.00)          | 0.000113 (Mann-Whitney U Test) |
| Location | Right colon                | 1/19 (5.3%)                | 1.000000 (Fisher’s Exact Test) |
| PNI +    | 11/19 (57.9%)              | 7/11 (63.6%)               | 0.000317 (Fisher’s Exact Test) |
| OCT3/4 + | 14/19 (73.7%)              | 3/11 (27.3%)               | 0.022746 (Fisher’s Exact Test) |
| Tumor front | Expansive (94.7%)       | 1/19 (9.1%)                | 0.000004 (Fisher’s Exact Test) |
The study also shows that loss of E-cadherin significantly correlated with increased tumor grade, increased tumor budding, higher N categories and invasive tumor front. OCT3/4, a stem cell marker that has been involved in EMT, indeed showed significant correlation with morphological clues of EMT such as tumor buds and invasive type front, but also with tumor grade and number of positive lymph nodes. The findings of the present study have also demonstrated that the alteration of expression of Vimentin, E-cadherin and OCT3/4 is absent in normal tissues, being only observed in tumors, which strongly suggests they can be used as biomarkers for aggressive disease.

Vimentin expression has been shown to be consistently associated with the EMT and thus with prognosis in various types of cancer, including breast carcinoma, head and neck squamous cell carcinoma, gastric adenocarcinoma, non small cell lung carcinoma, prostate adenocarcinoma and COAD(41-45). It has been shown that high Vimentin expression, together with another EMT transcription factor (Slug) is associated with lymph node
metastasis and poor prognosis in CRC (46). These findings recommend Vimentin as a biomarker for aggressive tumors. Also, we should not underestimate the role that Vimentin can have in therapy. One study showed that miRNA-17-5p directly binds to the 3′UTR of VIM mRNA and it inhibited the metastasis of CRC into liver in vivo (47). Another study showed that the FiVe1 compound (FOXC2-inhibiting Vimentin effector-1) can selectively and irreversibly inhibit the growth of breast cancer cells during EMT and soft tissue sarcomas, by directly interacting with Vimentin, leading to a more epithelial state in mesenchymally transformed cells (48).

E-cadherin abnormal expression has been also extensively studied and its loss of expression has become synonymous with the EMT. The loss of E-cadherin is correlated with infiltrative tumor growth and lymph node metastasis in colon cancer (49). In hepatocellular carcinoma an association of loss of E-cadherin with adverse clinicopathological factors and prognosis has been demonstrated (50). In cervical cancer loss of E-cadherin is an independent prognostic factor (51). Besides, it can be targeted by small molecules that can restore expression and enhance the activity of cell-to-cell adhesions, with the potential of inhibiting
metastasis, being a candidate for targeted therapy (52).

OCT3/4 is a major regulator of differentiation, pluripotency, and self-renewal in embryonic stem cells (53). Its role in cancer is to confer “stemness” to cancer cells, contributing to carcinogenesis and metastasis, leading to poor outcomes (54). It has been found to be overexpressed in a variety of cancers such as colon adenocarcinoma, lung adenocarcinoma, prostate adenocarcinoma, cervical and breast carcinoma (55-59). It has been found to promote the EMT in hepatocellular carcinoma through activation of Snail signaling (60). Its role in cancer is to confer "stemness" to cancer cells, contributing to carcinogenesis and metastasis, leading to poor outcomes (54). It has been found to be overexpressed in a variety of cancers such as colon adenocarcinoma, lung adenocarcinoma, prostate adenocarcinoma, cervical and breast carcinoma (55-59). It has been found to promote the EMT in hepatocellular carcinoma through activation of Snail signaling (60).

Conclusions

In summary, the expression of Vimentin and loss of E-cadherin are independently correlated with clinicopathological characteristics that are well-established adverse prognosis factors. OCT-4 is also highly expressed COAD tissue, and is a potential biomarker of epithelial to mesenchymal transition. Specific therapy against Vimentin, E-cadherin and tumor stem cells may block the EMT, which is a promising target of colon cancer treatment, and can assist in the clinical treatment of COAD. In daily anatomical pathology practice, the immunoprofile of COAD might have a prognosis impact. Further, larger, studies are necessary to check the prognosis role of EMT associated markers.

Conflict of Interest

The authors declare no conflict of interest.

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Ethical Statement

Patient consent was waived due to the retrospective nature of this study. The study protocol was approved by the Ethics Committee of “Prof. Dr. Alexandru Treistorianu” Oncological Institute, Bucharest, Romania.

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