ANTICANCER RESEARCH *38*: 811-816 (2018)
doi:10.21873/anticanres.12288

The Mesenchymal–Epithelial and Epithelial–Mesenchymal Cellular Plasticity of Liver Metastases with Digestive Origin

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Abstract. *Background: Few data are available regarding the epithelial to mesenchymal transition (EMT) /mesenchymal to epitheilal transition (MET) in the liver metastasis of digestive cancers. The aim of this study was to establish EMT/MET metastatic tumor cell plasticity according to the histological growth pattern of liver metastases. Materials and Methods: Biopsies from 25 patients with liver metastasis (desmoplastic, replacement and pushing type) were evaluated. Double immunostaining of E-cadherin/vimentin, keratin 8,18/vimentin and E-cadherin/ keratin 8,18 were performed. Results: The following cell types were noted: epithelial, mesenchymal, non-differentiated and differentiated hybrid mesenchymal/ epithelial and non-hybrid phenotype. All cases had mesenchymal/ epithelial phenotype cells. A significant correlation was found between the non-differentiated hybrid mesenchymal/ epithelial phenotype metastatic cells and histological growth pattern for gastric and colorectal cancer. Conclusion: A MET-targeting strategy, in conjunction with conventional chemotherapy, may be useful for the treatment of liver metastases.*

The epithelial to mesenchymal transition (EMT) represents the conversion of epithelial cell phenotype to mesenchymal cell phenotype, which is characterized by elongated, spindle-shaped cells. The mesenchymal to epithelial transition (MET) is the reverse phenomenon. There are three cell phenotypes described in the EMT/MET process: epithelial, mesenchymal and a hybrid epithelial/mesenchymal (partial or intermediate EMT) phenotype (1).

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Key Words: Epithelial–mesenchymal transition, mesenchymal–epithelial transition, liver metastases.

EMT was described in embryogenesis and organ development in physiological situations (2-4). In malignant lesions, EMT involves cytoskeletal disorders, loss of cell–cell adhesion and apical-basal cell polarity. In colorectal carcinoma, EMT was found in the cells from the invasive front (5). In pancreatic adenocarcinoma, loss of E-cadherin expression was noted in well- and poorly differentiated ductal adenocarcinoma, and few or none of the undifferentiated carcinomas (6, 7). Fewer than 50% of gastric cancer cells express E-cadherin. It was shown that the diffuse-type gastric cancer is associated with the E-cadherin/N-cadherin switch, whereas the intestinal-type is related to up-regulation of transforming growth factor beta (TGF)β/loss of E-cadherin (8).

Data from the literature show, using murine experimental models of metastasis, the presence of MET phenomenon in liver, lung, and brain metastases (9-11). Liver metastases from prostate cancer showed epithelial morphology in most cases (12). In liver metastases of colorectal adenocarcinoma, the increased expression of E-cadherin and decreased vimentin expression was noted (13, 14). More recently, in an experimental model, the involvement of sciellin as an inducer of MET through the liver metastasis process of colorectal cancer was shown (15). It increased Wnt signaling and favored MET through the sciellin–β-catenin–E-cadherin axis. Thus, in both mouse and human pancreatic ductal adenocarcinoma, metastatic cells appear to re-acquire an epithelial phenotype with increasing lesion size. Immunohistochemical analysis revealed a higher immunoexpression intensity of claudin 7 in primary tumors than in micro-metastases. Increased immunoexpression of fibroblast-specific protein 1 was found in micro-metastases compared to gross metastases and primary tumors (16).

The replacement growth pattern was suggested as being prevalent in liver metastases with pancreatic origin (17) and the pushing type for the gastric origin (18). The following types of growth were described for liver metastases of colorectal cancer: replacement, pushing, desmoplastic and mixed. The prognostic role of the histological growth pattern

of liver metastasis was shown at diagnosis but also after portal vein embolization in colorectal cancer (19, 20).

The aim of this study was to establish metastatic tumor cell plasticity according to the histological growth pattern of liver metastases with colorectal, pancreatic and gastric origins.

Materials and Method

The present study included 25 patients who underwent surgery on between 2009-2016. Liver metastasis was of different origins: colorectal adenocarcinoma in 15 cases, pancreatic in seven cases and gastric adenocarcinoma in three cases. Signed consent was obtained from each patient included in this study.

All procedures were carried out according to the principles of the Declaration of Helsinki and were approved by the Institutional Review Board (no. 7339/22.04.2016). The patients included in the study underwent excisional tumorectomy of the liver metastasis. Metastatic fragments were fixed in 10% buffered formalin for 24 h and paraffin embedded. Morphological and immunohisto-chemical staining were performed.

Double immunostainings for keratin 18/vimentin and E-cadherin/vimentin were used. Heat-induced epitope retrieval with Bond Epitope Retrieval Solution 2 (ready-to-use, pH 9.0; Leica Biosystems, Newcastle Ltd, Newcastle upon Tyne, UK) for 20 minutes was the first step. Endogenous peroxidase blocking was achieved with 3% hydrogen peroxide for 5 minutes. The following primary antibodies were used: keratin 8,18 (monoclonal, clone 5 D3, ready to use), E-cadherin (monoclonal, clone 36B5, ready to use), vimentin (monoclonal, clone V9, ready to use). All antibodies used were from Leica Bond Biosystems. The Bond Polymer Refine Detection System and The Bond Polymer Refine Red Detection System were used for visualization. As chromogen, 3,3-diaminobenzidine dyhidrochloride was applied for 10 minutes, and hematoxylin was used as a counterstain for 5 minutes. The entire immunohistochemical procedure was performed with Leica Bond-Max (Leica Biosystems) autostainer.

The immunoreactivity was estimated as following: keratin 8,18/vimentin: red cytoplasmic/brown cytoplasmic, E-cadherin/vimentin: brown membranous/red cytoplasmic and E-cadherin/keratin 8,18: brown membranous/red cytoplasmic. Microscopic evaluation and image acquisition was performed with Axiocam 506 color (Zeiss, Jena, Germany). The mesenchymal/epithelial hybrid phenotype cells were quantified at ×400 magnification in three consecutive areas with highest density. For statistical analyses, SPSS 17 software (IBM Analytics, Armonk, NY, USA) was used; differences with $p=0.05$ were considered statistically significant.

Results

The microscopic evaluation of hematoxylin and eosin-stained liver metastases of digestive origin revealed three histological growth patterns, as following: desmoplastic (28%), pushing (32%) and replacement (40%). All of the liver metastases of pancreatic origin included in the study had a replacement growth pattern, while those with gastric origin had the pushing growth pattern. The colorectal liver metastases exhibited all three histological growth patterns. Most of the liver metastases described above had G2 tumor grade (48%), followed by G3 (44%) and G1 (8%).

Double immunostaining for E-cadherin/vimentin and keratin 8 or 18/vimentin revealed the following cell types for the desmoplastic (seven cases), pushing (five cases) and replacement histological growth pattern of CRCLM (three cases): epithelial phenotype: E-cadherin$^+$/vimentin$^-$, keratin 8,18$^+$/vimentin$^-$ metastatic cells; mesenchymal phenotype: E-cadherin$^-$/vimentin$^+$, keratin 8,18$^-$/vimentin$^+$; mesenchymal/epithelial or non-differentiated hybrid phenotype: E-cadherin$^+$/vimentin$^+$, keratin 8,18$^+$/vimentin$^+$; and non-hybrid phenotype: E-cadherin$^-$/vimentin$^-$, keratin 8,18$^-$/vimentin$^-$ (Figure 1a). A heterogeneous expression of E-cadherin, keratin 8,18 with values ranged between 1 to 3 for epithelial phenotype metastatic cells was present. The mesenchymal/epithelial hybrid non-differentiated phenotype cells were noted inside of the areas with decreased E-cadherin, keratin 8,18 expression. All liver metastases presented hybrid phenotype cells. The distribution of hybrid non-differentiated phenotype cells was isolated or in clusters for desmoplastic (Figure 1b and c) and replacement types, and isolated for the pushing type (Figure 1d).

The same heterogeneous patterns of E-cadherin and keratin 8,18 expression were noted in the primary tumors of desmoplastic, pushing and replacement types of colorectal cancer liver metastases. A tendency for non-differentiated hybrid type tumor cells to localize in the basal part of the glands (desmoplastic type) and in the basal (Figure 1e), intermediate and luminal part of the glands (replacement type) was noted. In the primary tumor of replacement model, the luminal hybrid non-differentiated cells (E-cadherin$^+$/vimentin$^+$; keratin 8,18$^+$/vimentin$^+$) had cellular shape changes, such as hybrid ameboid/mesenchymal morphology (Figure 1f). One desmoplastic type corresponding primary tumor was characterized by the absence of hybrid non-differentiated tumor cells.

In the replacement and pushing histological growth pattern of liver metastases and corresponding primary tumors with pancreatic (seven cases) and gastric (three cases) origin, the heterogeneous profile of E-cadherin/vimentin, keratin 8,18 immunoexpression was maintained. The distribution of hybrid phenotype cells was isolated for pushing type liver metastases growth pattern (gastric; Figure 1g) and isolated or clusters for replacement types (pancreatic origin) types.

The relation between the arithmetic averages of hybrid non-differentiated cells the distribution pattern in primary tumors and the histological growth pattern of liver metastases are summarized in the Table I.

The double immunostaining of E-cadherin and keratin 8/18 revealed the expression of keratin 8/18 to be predominant in hepatic-metastatic cells of colorectal, pancreatic and gastric cancer. The distribution of co-expressing E-cadherin$^+$/keratin 8,18$^+$ cells (hybrid differentiated phenotype) was isolated for pushing type

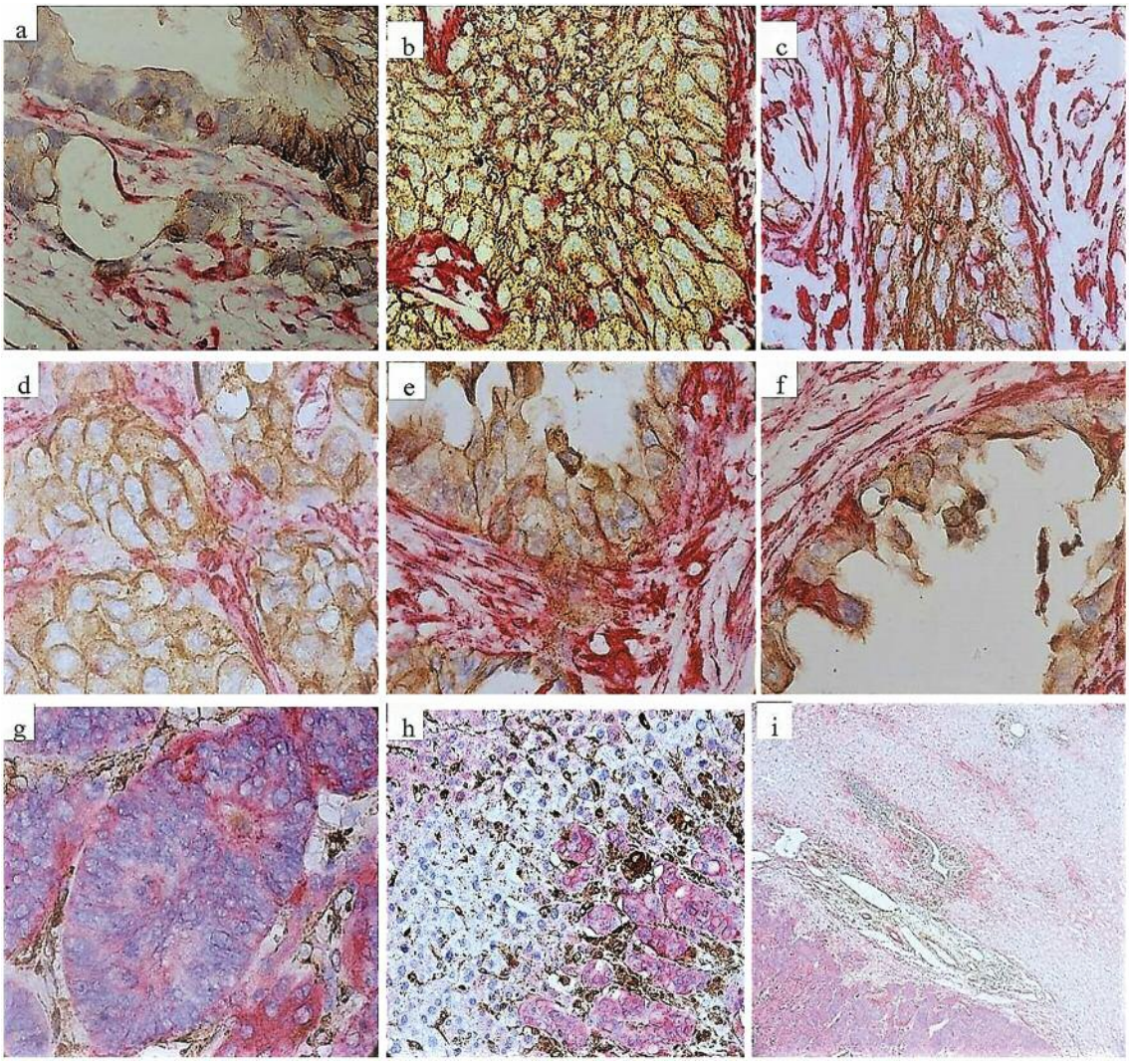

Figure 1. *Different patterns of epithelial to mesenchymal transition in liver metastases of various origins. a: Non-hybrid phenotype cells, E-cadherin^{-}/vimentin^{-}, in the replacement growth pattern of colorectal liver metastases (CRCLM) E-cadherin/vimentin double immunostaining, ×1,000 magnification. b: Isolated cell with hybrid phenotype in desmoplastic type of CRCLM, E-cadherin/vimentin double immunostaining, ×400 magnification. c: Clusters of hybrid cells, desmoplastic type of CRCLM, E-cadherin/vimentin double immunostaining, ×400 magnification. d: Isolated hybrid phenotype cells in the pushing type of CRCLM, E-cadherin/vimentin double immunostaining, ×400 magnification. e: Isolated cell basal hybrid phenotype in the replacement type CRCLM, E-cadherin/vimentin double immunostaining, ×400 magnification. f: The amoeboid/mesenchymal morphology of hybrid phenotype cell in the replacement type of CRCLM, E-cadherin/vimentin double immunostaining, ×1,000 magnification. g: Isolated hybrid phenotype cell in the pushing type CRCLM, keratin 8,18/vimentin double immunostaining, ×1,000 magnification. h: Keratin 8,18 expression in metastatic epithelial phenotype cells and hepatocytes in the replacement type of a pancreatic liver metastasis keratin 8,18/vimentin double immunostaining, ×400. i: Keratin 8/18 immunostaining with intensity value of 3 in hepatocytes close to the portal spaces, keratin 8,18/vimentin double immunostaining, ×200 magnification.*

(colorectal and gastric) and isolated or clustered cells for replacement and desmoplastic growth pattern of liver metastases (colorectal and pancreatic) types.

Regarding the E-cadherin/vimentin and keratin 8,18/vimentin immunoexpression at the border between metastases and the adjacent liver, all of the cases had higher intensity of E-cadherin and keratin expression in the metastatic epithelial phenotype cell compare with hepatocytes (Figure 1h), with a reduced intensity in the hepatocytes close to metastases, but with increased intensity at a distance from them. In cases with tumor invasion of the portal space, the hepatocytes in close vicinity to the portal space had high intensity of keratin 8/18 and E-cadherin immunoexpression, independent of histological growth pattern of liver metastases (Figure 1i).

TabIe I. *The relation between the arithmetic average of hybrid non-differentiated cells, histological growth pattern of liver metastases and the distribution pattern in primary tumors and liver metastases.*

Histological growth pattern of liver metastases/ primary tumor	Hybrid non-differentiated cells in liver metastases (E-cadherin$^+$/vimentin$^+$/ primary tumor)		Hybrid non-differentiated cells (keratin 8,18$^+$/vimentin$^+$/ primary tumor)		The distribution pattern of hybrid non-differentiated cells in liver metastases and primary tumors	
CRCLM, desmoplastic type	3.14	2	3.71	2.85	Isolated or cluster	Isolated or clusters, predominantely in the basal region of the glands
CRCLM, pushing type	2.6	2.8	1.2	1.6	Isolated	Isolated
CRCLM, replacement type	4.33	4.66	3.33	6.66	Isolated or clusters	Isolated or clusters; basal, intermediate and luminal part of the glands; hybrid, ameboid/mesenchymal changes
PLM, replacement type	2	4.85	3	5.42	Isolated or clusters	Isolated or clusters
GLM, pushing type	4	7	4	5	Isolated	Isolated

CRCLM: Colorectal liver metastases, PLM: pancreatic liver metastases, GLM: gastric liver metastases.

For the primary colorectal cancer tumors, significant correlation was found between the presence of non-differentiated hybrid phenotype cells (E-cadherin$^+$/ vimentin$^+$; keratin 8,18$^+$/vimentin$^+$) and the histological growth pattern (p=0.003; p=0.025). Significant correlation was noted between the number of non-differentiated hybrid phenotype cells (keratin 8,18$^+$/vimentin$^+$) and differentiated hybrid phenotype (keratin 8,18$^+$/E-cadherin$^+$) in primary tumors, and those in liver metastases: p=0.009 and p=0.044, respectively. A significant correlation was noted between the number of differentiated hybrid phenotype cells (E-cadherin$^+$/keratin 8,18$^+$) and of non-differentiated hybrid phenotype cells (keratin 8,18$^+$/vimentin$^+$, p=0.05; E-cadherin$^+$/vimentin$^+$, p=0.032) in liver metastases. A similar association was present in primary tumor (p=0.050).

For pancreatic cancer, a significant correlation was found between the number of differentiated hybrid phenotype cells and differentiation grade G in primary tumor and corresponding liver metastases (p=0.05).

A significant correlation between the number of non-differentiated hybrid phenotype cells (E-cadherin$^+$/ vimentin$^+$) and the histological growth pattern characterized gastric cancer (p=0.005).

Discussion

In normal situations, E-cadherin, a calcium-dependent cell-adhesion molecule is necessary for epithelial histogenesis, tissue stabilization, differentiation and induction of EMT during embryogenesis (21, 22). In pathological situations, abnormal expression of E-cadherin and β-catenin favor the mesenchyme phenotype cell (23).

Keratin 8,18 expression in normal conditions was found in simple epithelium (liver, pancreas, kidney), mixed epithelium (breast, lung) and is involved in embryogenesis (24, 25). In pathological situations, its increased expression was noted in adenocarcinomas and squamous cell carcinoma with different localization (26, 27). The mains roles of keratin 8/18 were: modulation of protein localization, protein targeting and apoptosis (28).

Data from the literature show that MET is a part of the metastatic process, in which the tumor cells regain epithelial properties at their secondary site (29, 30). It was noted that metastatic lesions had the same features of epithelial immunoexpression markers as primary tumors (31, 32). This pattern was found in our study. The hybrid differentiated cell type (E-cadherin$^+$/keratin 8,18$^+$) was found in metastatic cells. Significant correlation between the presence of co-expressing E-cadherin/keratin 8/18 cells in primary tumor and colorectal cancer liver metastases was found also. These observations may support the idea that a constant number of hybrid differentiated cells from primary tumors migrate to the secondary organ and maintain the same phenotype there. The existence of significant correlation between the non-differentiated hybrid phenotype cells in primary tumor and differentiated hybrid phenotype cells in colorectal cancer liver metastases argues for EMT/MET and MET/EMT plasticity of some tumor cells. This hypothesis was sustained by a significant correlation between non-differentiated and differentiated hybrid phenotype cells in both primary tumor and liver metastases of colorectal carcinoma.

Strauss *et al.* showed that some cells with hybrid epithelial/mesenchymal phenotype in primary ovarian

cultures and tumors in situ can be multipotent, express markers of other lineages, and drive tumor growth *in vivo* by giving rise to another epithelial/mesothelial subset as well as completely differentiated epithelial cells (33). Partial loss of E-cadherin expression was associated with carcinoma progression and unfavorable prognosis. In the final stages of numerous carcinomas, E-cadherin expression appears to be heterogeneous, with E-cadherin$^-$ tumor cells interposed between E-cadherin$^+$ tumor cell areas, suggesting that certain carcinoma cells have EMT properties (34). The same heterogeneous phenotype cells, with E-cadherin$^+$/vimentin$^-$, E-cadherin$^+$/keratin 8,18$^+$, keratin 8,18$^+$/vimentin$^-$ cells between cells without immunoexpression of epithelial markers were noted in the liver metastases and corresponding primary tumors analyzed in the present study. Over 75% of circulating tumor cells in women with metastatic breast cancer were found to co-express epithelial marker, mesenchymal marker *N*-cadherin, and stem cell markers (35).

It was shown for the patients with replacement growth pattern of CRCLM the hazard of death was 2-2.5 times higher than for patients with pushing growth or mixed growth pattern, and nearly 4-times higher than for patients with desmoplastic growth pattern. The negative prognostic effect of the replacement growth pattern was even more pronounced after adjusting for tumor size (20). These findings correspond to the highest values of differentiated and non-differentiated hybrid cell types for the replacement histological growth pattern. A significant correlation was found between the histological growth pattern and frequency of cells with non-differentiated phenotype in colorectal and gastric cancer.

EMT was also observed in other cancer types and also in their corresponding metastases (36). Thus, EMT has already become a target for several different therapeutic agents tested in experimental or preclinical studies (37, 38).

Conclusion

This study suggests the existence of significant correlation between the presence of non-differentiated hybrid phenotype cells in primary tumor and differentiated hybrid phenotype cells in colorectal cancer liver metastases. These support the hypothesis of EMT/MET and MET/EMT plasticity of some tumor cells in liver metastases. The existence of significant correlation between the cells with non-differentiated hybrid phenotype and the histological growth pattern in colorectal and gastric cancer indicates the potential for an MET-targeting strategy, in conjunction with conventional chemotherapy, for treatment of liver metastases of digestive origin.

Conflicts of Interest

None declared.

Acknowledgements

The Authors are grateful to Patricia Berzava and Ciprian Onica for their excellent technical support.

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Received November 18, 2017
Revised January 1, 2018
Accepted January 4, 2018