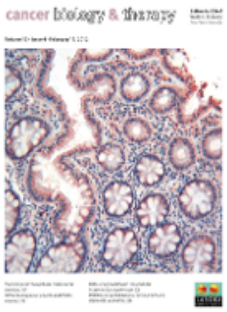

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RESEARCH PAPER

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Thymosin β-4 in colorectal cancer is localized predominantly at the invasion front in tumor cells undergoing epithelial mesenchymal transition

Sonia Nemolato,[1,*] Angelo Restivo,[3] Tiziana Cabras,[2] PierPaolo Coni,[1] Luigi Zorcolo,[3] Germano Orrù,[5] Mattia Fanari,[1] Flaviana Cau,[1] Clara Gerosa,[1] Daniela Fanni,[1] Irene Messana,[2] Massimo Castagnola,[4] Giuseppe Casula[3] and Gavino Faa[1]

[1]Dipartimento di Citomorfologia; Divisione di Anatomia Patologica; University of Cagliari; Cagliari, Italy; [2]Dipartimento di Scienze Applicate ai Biosistemi; University of Cagliari; Cagliari, Italy; [3]Dipartimento di Scienze Chirurgiche; University of Cagliari; Cagliari, Italy; [4]Dipartimento di Biochimica e di Biochimica Clinica; Catholic University of Rome; Rome, Italy; [5]Dipartimento di Chirurgia e Scienze Odontostomatologiche; Universita' degli Studi di Cagliari; Cagliari, Italy

Keywords: thymosins, $T\beta_4$, epithelial-mesenchymal transition, colorectal cancer, budding margins

Objective: Thymosin β-4 ($T\beta_4$) is a ubiquitous peptide that plays pivotal roles in the cytoskeletal system and in cell differentiation during embryogenesis. Recently, a role for $T\beta_4$ has been proposed in experimental and human carcinogenesis. This study was aimed at evaluating the correlation between $T\beta_4$ immunoractivity and colorectal cancer, with particular attemption to tumor cells undergoing epithelial-mesenchymal transition.

Methods and Results: 86 intestinal biopsies were retrospectively analyzed including 76 colorectal adenocarcinomas with evident features of epithelial-mesenchymal transition, and 10 samples of normal colorectal mucosa. Paraffin sections were immunostained for $T\beta_4$ and for E-cadherin. Total RNA was isolated from frozen specimens obtained, at surgery, from the normal colon mucosa, the deeper regions and the superficial tumor regions in four cases of colon cancer. $T\beta_4$ immunoreactivity was detected in the vast majority (59/76) of colon carcinomas, showing a patchy distribution, with well differentiated areas significantly more reactive than the less differentiated tumor zones. We also noted a zonal pattern in the majority of tumors, characterized by a progressive increase in immunostaining for $T\beta_4$ from the superficial toward the deepest tumor regions. The strongest expression for $T\beta_4$ was frequently detected in invading tumor cells with features of epithelial-mesenchymal transition. The increase in reactivity for $T\beta_4$ matched with a progressive decrease in E-cadherin expression in invading cancer cells. At mRNA level, the differences in $T\beta_4$ expression between the surrounding colon mucosa and the tumors samples were not significant.

Conclusions: Our data show that $T\beta_4$ is expressed in the majority of colon cancers, with preferential immunoreactivity in deep tumor regions. The preferential expression of the peptide and the increase in intensity of the immunostaining at the invasion front suggests a possible link between the peptide and the process of epithelial mesenchymal transition, suggesting a role for $T\beta_4$ in colorectal cancer invasion and metastasis.

Introduction

Epithelial-mesenchymal transition (EMT) is a complex process characterized by the loss of original epithelial features in embryonic and in tumor cells, associated with the gain of a mesenchymal phenotype and producing non-polarized isolated cells embedded in the extracellular matrix.[1] At molecular level, EMT requires multiple events, such as disruption of intercellular junctions, loss of cell polarity, microtubule disruption and basement membrane breakdown.[2] EMT has been originally described by embriologists as a key process in many developmental processes,[3] including the formation of the neural crest and of the myotome.[4] Recently, EMT has emerged to be a key step in cancer progression, allowing tumor cells to acquire an invasive behavior and disseminate.[5,6] EMT is now believed to be a major mechanism by which cancer cells become invasive, able to translocate from the initial neoplastic core, to penetrate vessel endothelium, entering circulation thus forming distant metastases.[7,8]

The typical hystologic expression of EMT may be observed at the infiltrative margins of carcinomas, as individual malignant cells, often acquiring a spindle shape, detach from the tumor mass, and stay independently within the interstitial matrix of the peritumoral stroma.[8] At immunohistochemistry, EMT is characterized by a dramatic decrease in intercellular expression of E-cadherin,[9] which was postulated to be the result of adherens junctions disruption.[10]

In colorectal cancer, EMT is evidenced by a change in tumor tissue architecture at the deep invasive tumor margins. This particular modification of cancer architecture has been referred as "budding margins," namely infiltrative margins with solid cell

*Correspondence to: Sonia Nemolato; Email: sonianemolato@libero.it
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nests formed by 2–3 cancer cells, which display their acquisition of motility by infiltrating the peritumoral connective tissue.[11]

Molecular studies have revealed that EMT consists of a number of cellular events, among which controlled basal membrane breakdown plays a crucial role. A new model for EMT mechanism has been proposed and new sequence in the distinct cellular steps which eventually lead to EMT has been evidenced.[12] In this model, microtubules, already known to have a central role in cell polarity and migration,[13] lose their stability, microtubule disruption causes basement membrane disassembly, disruption of the epithelial cell-basal membrane interaction and, eventually, breakdown of the basal membrane.[14] Interestingly, immunoreactivity for the anti-βtubulin antibody 6GT, which recognizes a sub-population of microtubules restricted at the basal regions of epithelial cells, progressively decreases in cells undergoing EMT, evidencing the destabilization of microtubules, followed by basal membrane disassembly and loss of epithelial characteristics by tumor cells.

Recently, $T\beta_4$ a member of a highly conserved family of 40–44 amino acid peptides that regulate actin polimerization by binding and sequestering monomeric G-actin,[15] has been hypotesized to trigger EMT in colorectal carcinoma by upregulating integrin-linked kinase (ILK).[16] Overexpression of $T\beta_4$ has been shown to upregulate ILK,[17] and consequently to cause the suppression of E-cadherin expression, resulting in disruption of adherens junctions and induction of EMT.[9]

In light of the foregoing data, focus of this study was to assess the pattern of immunoreactivity of $T\beta_4$ in colorectal cancer, and, in particular, the expression of this peptide in deep infiltrative margins in association with epithelial mesenchymal transition of cancer cells.

Results

Three main patterns of immunostaining for $T\beta_4$ were observed: a punctuated and granular cytoplasmic staining, localized in the entire cytoplasm or in basal or apical regions of enterocytes; a spot-like staining, localized in the perinuclear regions and representing, possibly, a localization of the peptide at the Trans-Golgi network; a homogeneous staining diffusely distributed in the entire cytoplasm. $T\beta_4$ expression was always restricted to the cytoplasm of normal, dysplastic and tumor cells. No nuclear reactivity was detected.

Normal colonic mucosa. All specimens of human colonic mucosa expressed $T\beta_4$: immunostaining for the peptide was observed in the superficial epithelium as well as in crypt epithelial cells (**Fig. 1A**). The intensity of reactivity for $T\beta_4$ was variable from a case to the next, ranging from a diffuse to a focal pattern. Immunostaining was observed in the cytoplasm of surface colon epithelium: it appeared homogeneous or punctate, and was mainly localized at the base of the cell or at apical cell regions, along the brush border. These different types of immunoreactivity for $T\beta_4$ were often found isolated. Occasionally, they were detected in the same cells (**Fig. 1A**). $T\beta_4$ was also found in granular deposits along the enterocyte surface or inside the intestinal lumen, spread over mucous secretion.

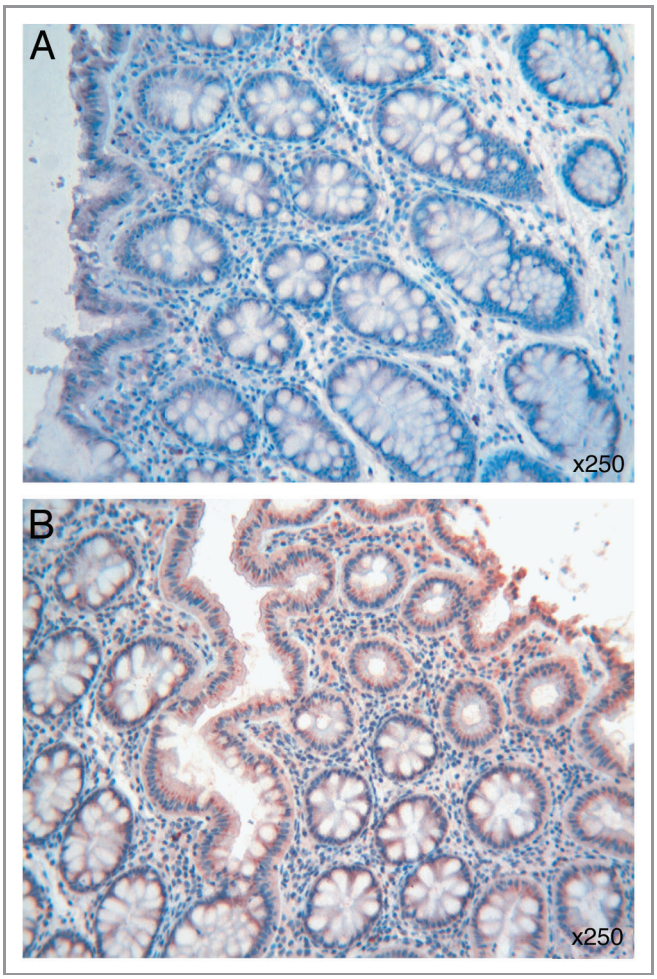

Figure 1. (A) A homogeneous and punctate immunostaining for $T\beta_4$ is detected in the cytoplasm at the base and at apical cell regions of normal colon epithelium. Immunoreactive granular deposits are observed on the enterocyte surface and inside the intestinal lumen. (B) $T\beta_4$ immunoreactive perinuclear spots are observed in the enterocytes of normal colonic mucosa adjacent to adenocarcinoma margins. OMx250.

Colonic mucosa surrounding adenocarcinoma. Substantial differences were observed in $T\beta_4$ immunoreactivity when samples of normal colonic mucosa were taken adjacent to adenocarcinoma margins: perinuclear spots, suggestive for a Golgi network localization and absent in colonic mucosa distant from the tumor, appeared instead prominent in the majority of enterocytes in proximity of tumor cells (**Fig. 1B**).

Adenocarcinoma. $T\beta_4$ expression was detected in 32 out of the 46 colorectal adenocarcinomas, with differences both in the type of reactivity and in intensity. Two main patterns of immunostaining were found in colorectal adenocarcinoma: a mild fine granular reactivity and a spot-like perinuclear staining, which appeared particularly evident in tumor cells with glandular arrangement (**Fig. 2A**). Striking differences in $T\beta_4$ expression were also found inside the same tumor: in the parallel investigation of well differentiated and less differentiated or undifferentiated areas, we observed an heterogeneous distribution of the peptide, characterized by a decrease in intensity proceeding from

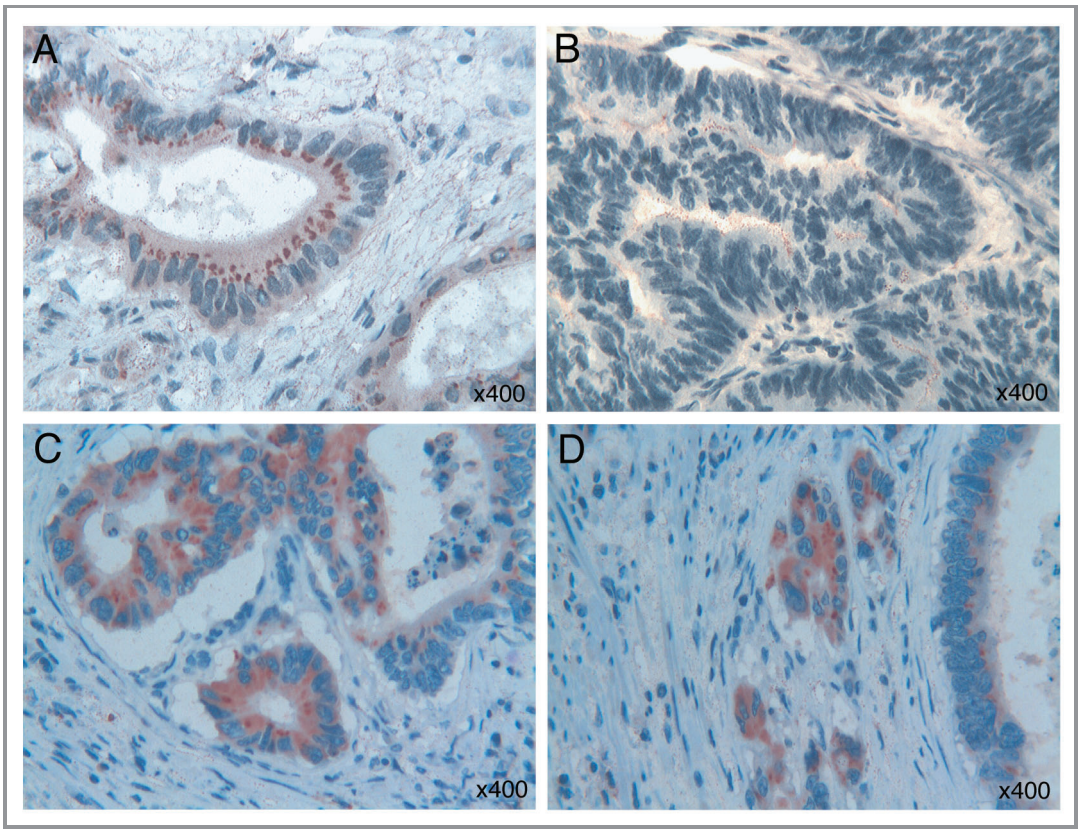

Figure 2. (A) A fine granular $T\beta_4$ reactivity and a spot-like perinuclear $T\beta_4$ immunostaining are observed in colorectal tumor cells with glandular arrangement. (B) Fine granular intraluminal immunoreactivity for $T\beta_4$ is detected in less differentiated tumor areas. (C) Strong immunoreactivity for $T\beta_4$ in the "budding margins" of colon cancer. (D) Strong cytoplasmic immunoreactivity for $T\beta_4$ in isolated infiltrating tumor cells with features of epithelial-mesenchymal transition. OMx400.

Grade 1 to Grade 3 zones, with strongly immunoreactive areas adjacent to negative tumor zones. In less differentiated tumor zones, we frequently observed an intraluminal immunoreactivity for $T\beta_4$ (**Fig. 2B**). Immunostaining for $T\beta_4$ was constantly restricted to the cytoplasm of tumor cells; no nuclear expression of the peptide was found. Moreover, in all 32 immunoreactive cases, we observed a positive trend in $T\beta_4$ reactivity from superficial areas toward deeper tumor regions, at the invasive front. The highest degree of immunoreactivity for $T\beta_4$ was always found in deepest areas of adenocarcinomas, at the invasive front with budding margins. The highest levels of reactivity for $T\beta_4$ were detected in the cytoplasm of isolated infiltrating tumor cells undergoing EMT (**Fig. 2C and D**).

To further elucidate the significance of $T\beta_4$ expression in the process of EMT in colon cancer cells, the status of cell-to-cell adhesion was evaluated by immunostaining for E-cadherin. As expected, a diffuse and strong membranous stainins was noted in normal mucosa. Immunostaining for E-cadherin was maintained in the central areas of adenocarcinoma. Significantly, E-cadherin immunoreactivity showed a decrease in intensity with fragmentation of the membranous staining in tumor cells undergoing EMT (**Fig. 3**). As for mRNA expression detected by RT-PCR we didn't find any significant difference between normal colon mucosa and the deeper and superficial regions of colon cancer (**Fig. 4**).

Discussion

Thymosin β-4, a peptide named after its first detection in the calf thymus,[19] has been traditionally correlated with a relevant role in regulation of actin polymerization in living cells.[20] $T\beta_4$ has many other biological functions: it contributes to angiogenesis,[21]

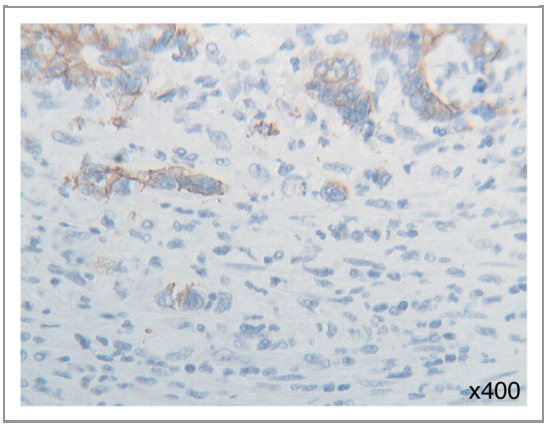

Figure 3. E-cadherin immunoreactivity: fragmentation of the membranous staining of tumor cells with features of epithelial-mesenchymal transition. OMx400.

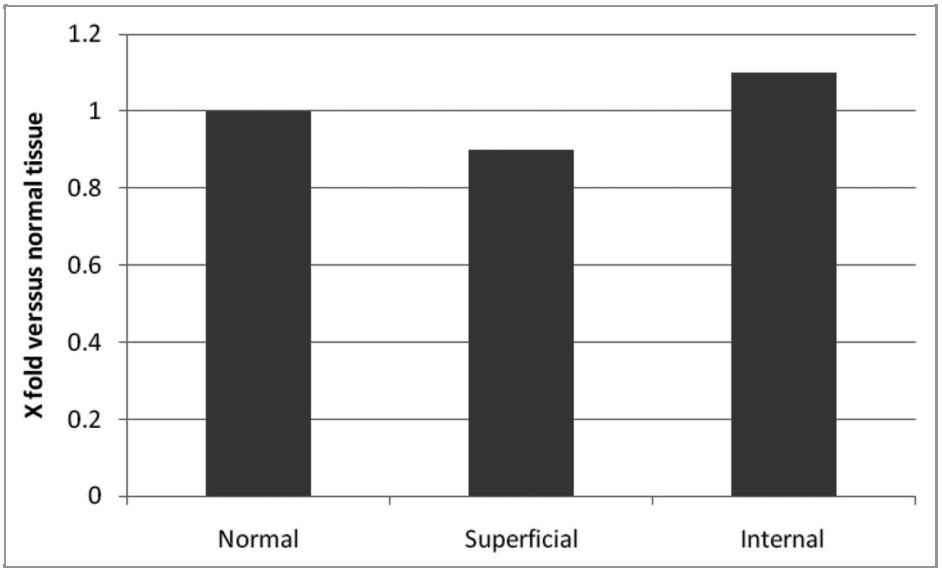

Figure 4. mRNA expression detected by RT-PCR reaction in four cases of colon adenocarcinoma.

cutaneous wound healing,[22] regulation of the inflammatory response[23] and promotion of cell migration.[17] $T\beta_4$ may also stimulate the AKT pathway, resulting in a strong anti-apoptotic effect on human cells[24] as well as in developing chick moto-neurons.[25] The antiapoptotic activity of $T\beta_4$ has been related to its ability to inactivate caspase-3, increasing cell survival rate.[26] More recently, $T\beta_4$ activity has been implicated in experimental and in human carcinogenesis.[27,28] This hypothesis was based on the observation of $T\beta_4$ effects on the cytoskeleton structure of cancer cells, on tumor cell motility, and on intra- and peritumoral angiogenesis. $T\beta_4$ has been recently detected in breast cancer, in few cases of colorectal cancers[29] and in urotelial carcinoma.[9] In some cases, $T\beta_4$ expression correlated with increased metastatic potential, thus providing a clue for the possible pro-metastatic role of the peptide.[30]

In this study, we clearly show that $T\beta_4$ is strongly expressed at the infiltrative front of colon cancer, particularly in the deep infiltrative margins, in tumor cells undergoing EMT (**Fig. 5**). This leads to the hypothesis that $T\beta_4$ might be involved in cancer progression, somehow favoring cancer invasion. In previous in vitro studies, transfection of $T\beta_4$ into a mouse melanoma cell line was shown to enhance metastatic potential of these cells, by the increase of intratumoral angiogenesis.[31] In a study on $T\beta_4$ expression in human breast cancer, the peptide production was found to be upregulated inside the tumor, and mainly focused on the intratumoral vascular component, reinforcing the hypothesis that $T\beta_4$ expression in tumor cells could be linked to angiogenesis.[29] In this study, we did not observe a significant immunoreactivity for $T\beta_4$ in intratumoral and in peritumoral vessels: $T\beta_4$ was always expressed in the cytoplasm of tumor cells, in the absence of any reactivity in the connective tissue, as previously reported by our group in developing human organs.[32,33] Outside the tumor cells, $T\beta_4$ immunostaining was restricted to peritumoral and intratumoral mast cells, a finding already reported in salivary gland tumors[34] and in human skin.[35]

Another intriguing finding in our study is the observation of a progressive decrease in the expression of E-cadherin on the cell surface in $T\beta_4$-reactive tumor cells undergoing EMT, with disappearance of E-cadherin in isolated spindle tumor cells migrating toward lymphatic and/or blood vessels. Since tumor cell dispersion relies on the loss of cell-cell adhesion, which is largely mediated by E-cadherin,[36] the strict association between $T\beta_4$ overexpression and E-cadherin decrease in invading colon cancer cells, identifies $T\beta_4$ as a powerful marker of EMT . On the basis of our data, we hypothesize that $T\beta_4$ may play a critical role in promoting EMT, leading to a deregulated cell-cell adhesion through E-cadherin down-regulation, as previously reported by others in urothelial carcinoma.[9] The absence of significant differences at mRNA level, between the normal colon mucosa and tumor tissues, may suggest a disregulation of $T\beta_4$ expression at post-transcriptional level.

A possible role for $T\beta_4$ in regulating motility and metastasis in non-small cell lung cancer,[37] in mouse fibrosarcoma,[38] in cultured colon cancer cells[10,16] and in human colon cancer cells[39] has been assumed in previous studies. Our observations of a preferential localization of $T\beta_4$ in infiltrating solid nests at the base of colorectal adenocarcinoma supports the possibility that $T\beta_4$ expression could modulate the invading activity of colorectal cancer cells with a similar role of the one played by βIII-tubulin at the invasive margins of colon cancer.[40] Our hypothesis confirms previous data in colon cancer cells coltures on a role of $T\beta_4$ as a trigger of a EMT in colo-rectal carcinoma.[41]

Given the recent reports on the ability of $T\beta_4$ to induce an embryonic reprogramming in adult cardiac progenitor cells, resulting in mobilization and differentiation to give rise to de novo cardiomyocytes,[42] we could speculate that $T\beta_4$ re-expression in tumor cells undergoing EMT might be the sign of an embryonic reprogramming in colon cancer cells, resulting in their mobilization and migration to give rise to distant metastases. According with this hypothesis, $T\beta_4$ could be considered as a possible target for future anticancer therapies.

In summary, in the present study we have demonstrated that $T\beta_4$ is frequently expressed in human colorectal adeno-carcinoma, with a marked preferential localization in tumor cells undergoing EMT, at the invasive front of the tumor. This evidence confirms previous hypotheses for a role of $T\beta_4$ in facilitating the progression of colon cancer.[28] Identifying the molecular mechanism underlying the intimate role of $T\beta_4$ in the process of EMT is a major challenge for future research with the prospective that inhibitors of this peptide might have a chance to become new therapeutic agents against colon cancer.

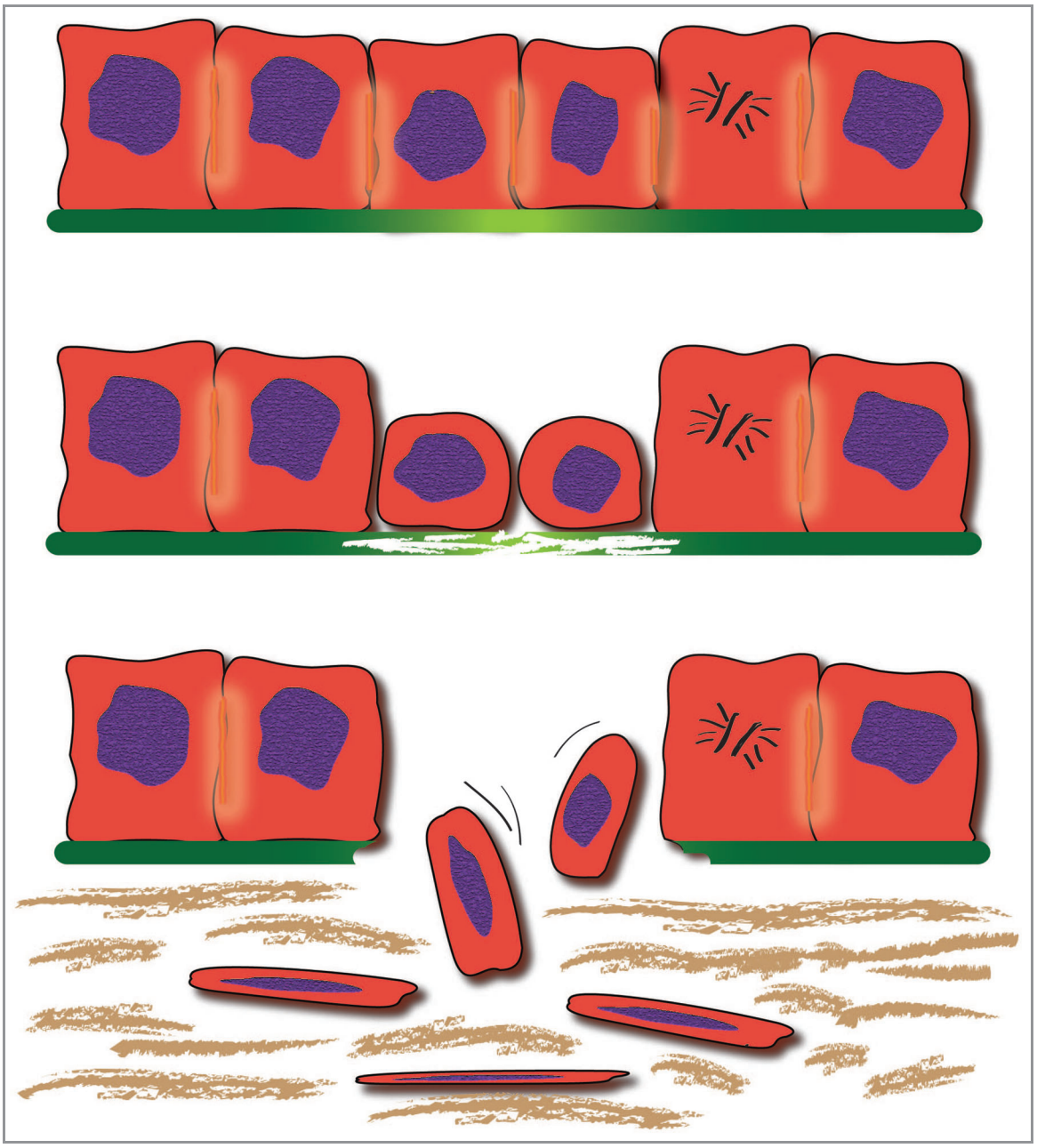

Figure 5. Sequential steps in epithelial-mesenchymal transition at the infiltrative margin of colon cancer. Some tumor cells detached from nearest cells acquire a spindle shape and infiltrate the surrounding tissues.

Patients and Methods

The study included archived paraffin-embedded colorectal sections obtained from 86 patients who underwent colonoscopy with biopsy or surgical colon resection. The cohort included 10 samples of normal colon mucosa and 76 colorectal adenocarcinomas. Colon cancers were included when characterized by budding margins with evident morphological signs of epithelial-mesenchymal transition.

Immunoistochemistry. For each tumor included in this study, two samples were immunostained for $T\beta_4$. Paraffin sections were immunostained with anti-$T\beta_4$ antibodies (Bachem-Peninsula Lab) and with anti-E-cadherin antibodies (Dako, A/S), using the labeled streptavidin-biotin complex system (LSAB2, Dako) in a Dako Autostainer (DakoCytomation). Briefly, slides were deparaffinized, rehydrated, and endogenous peroxidase activity was quenched (30 min) by 0.3% hydrogen peroxide in methanol. Slides were then subjected to heat-induced antigen retrieval by steaming unstained sections in Target Retrieval Solution (Dako TRS pH 6.1) for 30 min. Slides were incubated with 10% normal goat serum in phosphate-buffered saline (PBS) for 60 min to block non-specific binding, followed by incubation (60 min at room temperature) with the monoclonal anti-Thymosin β-4 and anti E-cadherin antibodies, diluted 1:100 in blocking solution.

Slides were extensively washed with PBS containing 0.01% Triton X-100 and incubated with a secondary reagent (En Vision kit) according with the manufacturer's (Dako) instructions. Diaminobenzidine (DAB) was used as chromogen. After additional washes, color was developed using the AEC reagent (Dako); sections were counterstained with Mayer's hematoxylin and mounted. Sections of reactive lymph nodes with $T\beta_4$-immunoreactive histiocytes were utilized as positive control for the immunohistochemical reaction. As negative control, the same procedure was applied omitting the primary antibody. All cases were independently reanalyzed by two pathologists specialized in gastrointestinal pathology (SN, GF), according to the 1999 WHO classification. The study protocol was approved by the Institutional Review Board.

Real Time RT-PCR. Tissue samples, obtained after surgery, from the surrounding colon mucosa, from the superficial and from the deeper tumor margins, were immediately frozen in dry ice, and kept at -70°C until lysis for RNA extraction. Total RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen) according to manufacturer's instructions.

As internal control was used the human β actin gene. The following primers (β-act F. = 5'-GCATGGGTCAGAAGG-3', β act R. = 5'-AGGCGTACAGGGATAG-3', tb4 F. = 5'-GGCC-ACTGCGCAGACCAGACT-3' tb4R. = 5'-CTTGATCCAACC-TCTTTGCATCTTACAA-3') were designed using the sequences of the Tymosin β-4 min RNA (GenBank accession no. NM_001101) and the human β actin mRNA (GenBank accession no. NM_001101).

Real-time reverse-transcriptase PCR analysis was perfomed in a Light Cycler apparatus (Roche) with a LightCycler-RNA Amplification kit SYBR Green I (Roche Diagnostics) according to the manufacturer's instructions. The 20 µl final volume contained: 3 mM MgCl2, 0.25 µM of each primer 2 µl of RNA extract. Cycling was performed using the following amplification conditions: an initial reverse transcription at 55°C for 10 min, denaturation at 95°C for 30 sec followed by 35 cycles at 95°C for 10 sec, 53°C for 10 sec and 72°C for 8 sec with subsequent melting analysis: heating to 95°C for 20 sec, cooling to 45°C for 10 sec and reheating to 95°C at a rate of 0.2°C per second. Fluorescence was detected at the end of the 81°C segment in PCR step (single mode) and at 45°C segment in the melting step (continuous mode) in the F1 channel. The relative gene expression was analyzed by using the 2-ΔΔCT method.[18] For each analysis, three distinct biological replicas were done, and quantitative data were expressed as mean. Values of fold change in tb4 gene expression relative to the β actin RNA to above 2 or below 0.5 were considered significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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