

# Immunoglobulin M Distribution and Modular Peptide Interactions in the Stroma of Human Colorectal Adenomas and Adenocarcinomas

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### Abstract

Background: Tissue organisation field theory explains that cancer starts as a result of an alteration in supporting tissue and inflammatory cells. The immunoglobulins (Igs) detected in colorectal adenocarcinomas come from the loss of integrity of resident µ chain-producing cells. Nearly half of the contact residues of Igs are aromatic and highly reactive. In the same way, the vast majority of cell surface proteins in the extra-cellular matrix contain a number of different domains or modules. The (Arg-Gly-Asp) RGD receptor domain of fibronectin constitutes cell adhesion receptors for cell-matrix adhesion and for bidirectional signaling across the membrane. Highly homologous oligopeptides emulate and compete with matrix adhesive proteins: Streptavidin binds to cells via the (Arg-Tyr-Asp) RYD mimetic RGD peptide. Furthermore, tyrosine-tryptophan-threonine-aspartic acid (YWTD) domains, physiologically binds laminin and seven different endocytic receptors contain 1-8 YWTD beta-propeller domains.

**Objective:** The primary aim of this study was to evaluate the in situ presence and distribution of  $\mu$  chains in 46 colorectal tumors of different histological grades using fluoresceinate goat anti-human µ chains. The secondary aim was to evaluate the cell and stromal interactions of the fluoresceinate YWTD, RGD antigens and streptavidin in sequential biopsy specimens of the same samples.

Results: The detection of µ chains was low in the adenomas and high in the adenocarcinomas. Two morphological types of B cells differently associated with tissue integrity. Only stromal µ chain-producing cells strongly bound YWTD, RGD and streptavidin.

Conclusion: In colorectal tumors, RGD-mimicking site peptides mainly compete with fibronectin/immunoglobulin binding. The presence of µ chain/YWTD interactions shows that sequences richer in aromatic residues should be considered.

Keywords: Lymphocyte homing; YWTD; RGD; Colorectal cancer; Streptavidin; Fibronectin

Abbreviations: APC: Adenomatous Polyposis Coli; BCR: B Cell Receptor; ECM: Extra-Cellular matrix; EGF: Epidermal Growth Factor; FN: Fibronectin; FZD: Frizzled Family; GSK: Glycogen Synthase Kinase; IFI: Immunofluorescence; IFNy: Interferon y; Igs: Immunoglobulins; IH: Immunohistochemistry; IPCs: Immunoglobulin-Producing Cells; LRP6: Low-Density Lipoprotein (LDL) Receptor Protein; MIDAS: Metal Ion-Dependent Adhesion Site; MMPs: Metalloproteinases; PHSRN: Pro-His-Ser-Arg-Asn Synergy Site; RGD: Arginine-Glycine-Aspartic Acid; RYD: Arginine-Tyrosine-Aspartic Acid; TAMs: Tumor-Associated Macrophages; Th: T Helper; Wnt: Family of Cysteine-Rich, Secreted Glycoprotens; YWTD: Tyrosine-Tryptophan-Threonine-Aspartic Acid

# Introduction

Tissue organization field theory views tumor pathogenesis as a tissue phenomenon linked to alterations in stromal/epithelial interactions [1-3]. Accumulating evidences suggest the existence of protein-protein ligand binding among cytoskeletal proteins and a various signaling molecules, thus changing the number and strength of the bonds between adhesion receptors and extra-cellular components [4].

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Human neoplastic foci in vivo contain immunoglobulin-producing cells (IPCs) that participate in local transformations. We have found that the immunoglobulins detected in neoplastic colorectal lesions do not come from circulating cells or a remote control mechanism, but from the loss of integrity of resident cells expressing IgM that are morphologically different from mature B cells [5]. Nearly half of the contact residues in the antibodies are aromatic and this is often found in antibody recognition sites. Analyses of tyrosine and tryptophan residues have demonstrated that these amino acids are more exposed to solvent than those in the framework of the variable domains and other

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proteins, something that makes them available for ligand interaction [6]. In the same way, the vast majority of proteins on cell surfaces and in the extra-cellular matrix (ECM) contain a number of different domains or modules arranged in tandem on a single polypeptide chain. These modules form the basis of cell adhesion, which involves multi-molecular protein complexes of trans-membrane adhesion receptors anchoring intracellular cytoskeletal structural proteins and signal transduction molecules [7].

One of these modules, the (Arg-Gly-Asp) RGD receptor domain of fibronectin (FN), has attracted widespread attention. Fibronectin is a stromal adhesive protein that binds to the  $\alpha 5\beta 1$  integrin located on the surface of cell membranes by means of an independent cell adhesive region that requires two critical amino acid sequences that function in synergy to ensure optimal binding: An Arg-Gly-Asp (RGD) sequence and a Pro-His-Ser-Arg-Asn (PHSRN) sequence [8]. The striking aspect of integrin-ligand interactions is that they can be dynamically regulated: Integrins can exist in inactive or active states, but only those the active state can to bind ligands [9]. The unliganded structure of integrin takes on what is called a "closed" conformation and the >90% of particles in the closed conformation confirm that this is the most stable conformation [10]. High-affinity ligand binding requires integrins to become "activated" by undergoing conformational changes regulated by inside-out signals and, in turn, integrin ligation triggers outsidein signals that, among other responses, regulate cell motility and gene expression [11]. These two (inside-out and outside-in) mechanisms may be intrinsically linked because there is little evidence to indicate that their conformational changes are different [12].

Acquisition of the active state requires bivalent cations and is accompanied by conformational changes involving both  $\alpha$  and  $\beta$  subunits. These two domains are partly overlapping in the inactive state [9] and integrin activation may reveal the  $\beta$ 1 stalk region by triggering a conformational shift of  $\alpha$ 5 in relation to  $\beta$ 1 [13]. Moreover, fibronectin ligand binding sites within integrins have an enhanced propensity to cluster and interact with the cytoskeleton upon ligand binding, thus indicating that both biochemical ligand binding and biophysical receptor clustering are needed to engage interactions with a full complement of cytoskeletal and signaling proteins [14].

Integrin recognition of RGD is divalent cation dependent and conformationally sensitive, with manganese and magnesium generally promoting binding and calcium having the opposite effect [9]. The positively charged arginine and negatively charged aspartic acid side chains of the RGD tri-peptide motif bridge integrin at the center of the ligand-binding pocket by means of electrostatic interactions [10]. The cation binding site has been christened MIDAS, which stands for metal ion-dependent adhesion site. This conserved mode of ligand binding explains the propensity of a given integrin to bind to multiple ligands and the ability of a given ligand to be recognized by multiple integrins, thus allowing integrins to fulfill the well-established pharmacological principles of agonism, partial agonism, antagonism and efficacy [15].

Highly homologous oligopeptides emulate and compete with matrix adhesive proteins [16] and competition studies of RGD peptides have shown that streptavidin binds to cells via an (Arg-Tyr-Asp) RYD mimetic RGD peptide and that this binding is independent of biotin recognition [17]. Immobilization of the protein (for example, by means of commercial streptavidin) alters the peptide's conformation and exposes the RGD-like site [18]. Streptavidin and peptide-based drugs incorporate peptide sequences similar to ligand recognition sequences in endogenous integrin ligands and thus compete for the ligand binding site within integrins [19].

In the stroma, the tyrosine-tryptophan-threonine-aspartic acid (YWTD) repeats are other modular proteins with a very reactive structure. The fact that half of their amino acids are aromatic makes them available for solvent exposure and the conserved Asp residue plays a number of crucial roles in defining and stabilizing the propeller structure of major membrane receptors. The YWTD repeats have become known as "YWTD spacers" and are shown in current reviews as squiggly lines that separate other domains. However, they may not only have important functions on their own, but may also have important orienting and architectural functions. YWTD repeats are the seventh most abundant extra-cellular repeats, a fact that underlines their evolutionary importance [20]. The YWTD repeats in nidogens seem to play an important role in ligand binding and have a compact, globular structure. They are also part of seven different endocytic receptors that contain from 1-8 YWTD beta-propeller domains and act as lipoprotein, vitellogenin and scavenger receptors [21].

YWTD repeats are also structural components of LRP6, a focal point controlling the Wnt/ $\beta$ -catenin pathway. The structure and function of the LPR6 ectodomain suggest a signaling platform consisting of a compact arrangement of four YWTD  $\beta$ -propeller/EGF domain pairs, a construction that supports the existence of interactions between LPR6 and its agonists or antagonists [22]. LRP6 acts as a co-receptor for the secreted WNT glycoproteins that have to bind to both frizzled (Frz) and LRP6 in order to turn on the canonical WNT pathway [23]. Structurally, these receptors form a single signaling complex and the function of WNT is to form a bridge between Frizzled and LRP [24]. This process is often perturbed in human colorectal cancers and other tumors as a result of the loss of the functional adenomatous polyposis coli gene product (Apc) or Axin or because  $\beta$ -catenin mutations prevent its phosphorylation and degradation, thus leading to constitutive Wnt- $\beta$ -catenin signaling [25].

The aim of this study was to evaluate the in situ presence and distribution of  $\mu$  chains in 46 spontaneous colorectal tumors of different histological grades using fluoresceinate goat anti-human  $\mu$  chains. We also evaluated the cellular and stromal interactions of streptavidin or fluoresceinate YWTD and RGD antigens in sequential biopsy specimens of the same samples.

# Materials and Methods

# Patients

The study involved a series of 46 sporadic colorectal adenomas and adenocarcinomas obtained from patients who underwent endoscopy or surgery at Rho Hospital and Milan's Luigi Sacco Hospital between January 2014 and July 2015. Twenty were from the colon (ascending, transverse and descending colon) and 26 from the distal intestinal tract (sigmoid colon and rectum). The samples were placed on formalinfixed, paraffin-imbibed tissue. The study was approved by the hospital's ASST Fatebenefratelli Sacco Buzzi Ethics Committee and the patients were enrolled after signing an informed consent form in accordance with the Declaration of Helsinki. None of the patients had undergone chemotherapy or radiotherapy before biopsy/surgery.

# Histology

All of the biopsy and surgical samples were formalin-fixed and paraffin-embedded, cut at 3  $\mu$ m and stained with hematoxylin and eosin. The adenomas were classified using the revised Vienna classification [26] as adenomas with low-grade dysplasia (10 cases) or adenomas with high-grade dysplasia (12 cases); serrated adenomas and mixed polyps together were considered adenomas. The colorectal

adenocarcinomas were graded using the current WHO classification [27], considering cancers with >95% gland formation as grade 1 (well differentiated), those with 50-95% gland formation as grade 2 (moderately differentiated) and those with <50% gland formation as grade 3 (poorly differentiated). Ten cases were graded G2 and 14 as G3; none was G1.

#### Tissues and fixation procedure

The tumor tissues used in this study came from surgically removed specimens placed on formalin-fixed, paraffin-imbibed tissue. Sections of 3  $\mu$ m were cut, mounted on poly-L-lysine-coated glass slides, pre-treated to enhance their antigenicity and de-paraffined using a standard method. Before the immunofluorescence (IFI) and immunohistochemistry (IH) investigations, they were fixed in methanol for 10 min, air dried and exposed to normal horse serum for 30 min at room temperature in order to prevent non-specific binding. After removing the blocking solution, the sections were incubated for one hour with PBS (PBS, BSA 1%, FBS 2%) in order to permeabilise the cells. To minimize the evaporation of the aqueous reagents from the specimen-containing slides, a coverslip parafilm was applied to the slide stainer.

### Immunofluorescence (IFI)

The slides were incubated with a  $\mu$  chain-specific goat anti-human IgM (FI-3020, Fluorescein anti-human IgM,  $\mu$  Chain Specific, diluted 1:100, Vector Laboratories, Burlingame, CA, USA), fluorescein tyrosine-tryptophan-threonine-aspartic acid, YWTD (FITC-YWTD diluted 1:100, Sellech, Houston, TX, USA) or fluorescein arginine-glycine-aspartic acid (Arg-Gly-Asp) RGD (FITC-Ahx-Arg-Gly-Asp dil 1:50, Sellech, Huston, TX, USA). While being protected from exposure to direct light at room temperature for 1 h, the samples were washed four times for five minutes in high-salt PBS (NaCL 4M and PO4 buffer) and mounted. Images of four randomly chosen fields of each sample were recorded at magnifications of 40x and 20x (Euroimmun, Lubeck, Germany).

# Immunohistochemistry (IH)

IH was carried out using the streptavidin immunoperoxidase method. The slides were incubated with horseradish peroxidase (horseradish peroxidase streptavidin, SA-5004, Vector, Burlingame, CA, USA. Dilution 1:50) for 1 h. After rapid washing with bi-distilled water, the staining reaction was performed in  $30 \,\mu$ L/mL 3,3'-diaminobenzidine tetrahydroxychloride DAB (Vector SK-4105, Burlingame, CA, USA) for 10 min. The slides were washed, counterstained with hematoxylin

and mounted using an aqueous mounting medium. Images of four randomly chosen fields of each sample were recorded at magnifications of 10x, 25x, 40x and 100x (VWR International, Leuven, Belgium).

# Microscopy

Fluorescence and optical microscopy of a minimum of ten randomly selected fields was performed semi-quantitatively by two pathologists blinded to the histological findings using a 20x and 40x objective lenses for fluorescence microscopy and 10x, 25x, 40x and 100x objective lenses for optical microscopy. The scoring of staining positivity and the semi-quantitative analysis of the morphological data were adapted from widely used criteria [28].

Fluoresceinate IgM staining intensity was graded 1-3: Focal, diffuse and highly diffuse. The percentage of stromal immunoglobulinproducing cells (IPCs) (in relation to all stromal cells), the percentage stromal B cells with irregular nuclei (in relation to all stromal B cells) and the percentage of  $\mu$  chain-positive membrane fragments (in relation to all  $\mu$  chain-positive components) were classified as none, <10%, >10-40% and >40%.

## Statistical analyses

The continuous variables (expressed as mean values and standard deviation) and categorical variables (expressed as absolute and relative frequencies) were compared using the chi-squared test. The data were analyzed using the Statistical Package for Social Sciences (SPSS 13.0, SPSS Inc., Chicago, IL, USA). All of the tests were two-sided and p values of <0.05 were considered statistically significant.

# Results

#### Study population

Table 1 shows the demographic and histological characteristics of the 46 patients.

# Semi-quantitative evaluation of fluoresceinate $\mu$ chains in colorectal adenomas and adenocarcinomas

The semi-quantitative study revealed a significant correlation between fluoresceinate  $\mu$  chain distribution and the histological severity of the tumors (p<0.001) (Table 2). Each grade of severity had its own histological features. All of the low-grade adenomas were characterized by a focal distribution of  $\mu$  chains that morphologically corresponded to a net but limited detachment of epithelial cells associated with small

	Low-grade dysplasia tubular adenoma (n=10)	High-grade dysplasia tubular adenoma (n=12)	Adenocarcinoma (n=24)	р
Age (years)	71.7 ± 8.6	62.5 ± 12.6	72.2 ± 10.6	
Gender				0.04
Males	6 (60.0%)	6 (50.0%)	16 (66.7%)	0.04
Females	4 (40.0%)	6 (50.0%)	8 (33.3%)	0.0
Colon localisation				0.6
Ascending	3 (30.0%)	1 (8.3%)	6 (25.0%)	
Transverse	2 (20.0%)	2 (16.7%)	4 (16.7%)	0.8
Descending	1 (10.0%)	1 (8.3%)	0	
Sigmoid/rectum	4 (40.0%)	8 (66.7%)	14 (58.4%)	

Table 1: Demographic and histological characteristics of the study patients.

IgM distribution	Low-grade dysplasia tubular adenoma (n=10)	High-grade dysplasia tubular adenoma (n=12)	Adenocarcinoma (n=24)	р
Focal	10 (100%)	0	0	
Diffuse	0	5 (41.7%)	0	<0.001
highly diffuse	0	7 (58.3%)	24 (100%)	

Table 2: Correlations between IgM distribution and histological features.

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aggregates of stromal  $\mu$  chain-positive cells (Figure 1); the remaining areas of the histological samples were not  $\mu$  chain-positive and had a well-preserved glandular structure, albeit with hyperplastic features and rich stromal cellularity.

A large proportion (41.7%) of the high-grade adenomas showed a more extensive loss of integrity and increased fluorescence (Figure 2) that also involved the preserved glandular tissue. The presence of  $\mu$  chains in the tissue retaining its structure was associated with little and localized loss of stromal tissue and the delimitation of vascular-like spaces (Figure 3). The fluorescence was diffuse.

The remaining 58.3% of the high-grade adenomas and all of the adenocarcinomas had a highly diffuse  $\mu$  chain distribution (Figure 4).

The fluorescence-delineated B cells were clustered or spread in the stromal tissue. The  $\mu$  chain-positive cells in the adenocarcinoma tended to lose their structural integrity and become fragmented.

# Morphological characteristics of IgM-producing cells in colorectal adenomas and adenocarcinomas

Direct microscopic evaluation allowed the different morphology





of  $\mu$  chain-positive cells in the biopsy specimens to be clarified. The fluoresceinate Ig anti- $\mu$  chains highlighted two main morphological types of B cells differently associated with tissue integrity: IPCs with a low cytoplasmic/nuclear ratio (Figure 5) and B cells with irregular nuclei (Figure 6). The former seemed to be related to epithelial cell membrane disintegration, whereas the IPCs with irregular nuclei were prevalently, but not exclusively, in tissue areas that had the structural



Figure 3: The presence of  $\mu$  chains in tissue that still maintains its structure is associated with a small loss of stromal tissue and the delimitation of vascular-like spaces (fluorescence microscopy, magnification 20x, detail).



**Figure 4:** Distribution of fluoresceinate anti-µ chain antibodies and fluoresceinate RGD and YWTD antigens in an adenocarcinoma (Fluorescence microscopy, magnification 20x).



**Figure 2:** High-grade adenomas show more extensive loss of integrity and increased interactions with fluoresceinate anti-µ chain antibodies and fluoresceinate RGD and YWTD antigens (Fluorescence microscopy, magnification 20x).



Figure 5: Fluorescence microscopy of IPCs with a low cytoplasmic/nuclear ratio (detail).

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characteristics of intestinal glands and an inflammatory appearance (Figure 7).

This confirms the presence of B cell populations whose plasticity and alternative functions differ from the classic paradigm of acquired and innate immunity. The IPCs with a low cytoplasmic/nuclear ratio were little represented in the low-grade adenomas (<10% of all stromal B cells), whereas they accounted for >40% of all stromal B cells in half of the high-grade adenomas and 83.3% of the adenocarcinomas. The increase in the proportion of these cells is therefore associated with a worsening in the histological grade of dysplasia (p<0.001) (Table 3).

The frequency and distribution of the cells with irregular nuclei



Figure 6: Immunofluorescence of B cells with irregular nuclei (detail).



**Figure 7:** IPCs with irregular nuclei are prevalent in tissue areas that have the structural characteristics of intestinal glands (Fluorescence microscopy; magnification 40x).

varied (Table 4). Seven of the ten low-grade adenomas did not have any B cells with irregular nuclei, whereas these accounted for 10-40% of all stromal B cells in 83.3% of the high-grade adenomas. Glandular tissue was well represented in such tumors: The destroyed area was larger than in the low-grade adenomas but once again had an intra-epithelial localization. The different deployment of the two B cell populations was very clear in the transitional zone between intact and destroyed tissue. B cells with irregular nuclei were less represented in the adenocarcinomas: They were not detected in 16.7% of the cases and accounted for <10% of all B cells in 62.5% of the samples.

In localized and limited areas of the examined tissues, we identified an unusual B cells morphology characterized by an accumulation of fluorescence on the membrane surface of individual cells with a low cytoplasmic/nuclear ratio (Figure 8). These B cells were not quantifiable and were localized in tissue areas with a loss of cellular integrity (Figure 9).

In three grade 3 adenocarcinomas, in which the total structural destruction also involved B cells, we only observed ICPs that had lost their integrity and appeared as membranous filaments exhibiting  $\mu$  chains (Figure 10). They were represented in 30% of the low-grade adenomas, in which their distribution was sporadic and they accounted for <10% of all  $\mu$  chains whereas, in 50% of the high-grade adenomas



Figure 8: Accumulation of fluorescence in individual IPCs (Fluorescence microscopy, detail).

IPCs	Low-grade dysplasia tubular adenoma (n=10)	High-grade dysplasia tubular adenoma (n=12)	Adenocarcinoma (n=24)	р
None <10% of stromal cells	0 10 (100%)	0 1 (8.3%)	3 (12.5%) 0	<0.001
10-40%of stromal cells >40% of stromal cellS	0 0	5 (41.7%) 6 (50.0%)	1 (4.2%) 20 (83.3%)	

Table 3: Correlations between the percentage of IPCs with a low cytoplasmic/nuclear ratio and histological features.

Irregular nuclei B cells	Low-grade dysplasia tubular adenoma (n=10)	High-grade dysplasia tubular adenoma (n=12)	Adenocarcinoma (n=24)	р
None	7 (70.0%)	0	4 (16.7%)	
<10% of stromal B cells	1 (10.0%)	1 (8.3%)	15 (62.5%)	10.001
10-40% of stromal B cells	1 (10.0%)	10 (83.3%)	5 (20.9%)	<0.001
>40% of stromal B cells	1 (10.0%)	1 (8.3%)	0	

Table 4: Correlations between the percentage of B cells with irregular nuclei and histological features.

and 100% of the adenocarcinomas their frequency was intermediate (10-40% of all  $\mu$  chains) or high (>40%) (Table 5) These data are significant because of the correlation between the histological severity of the tumors and the number of fragments (p<0.001). The fragments may be detected as individual membranous fragments (Figure 11) or concentrated on irregular membranous structures. The newly-formed membranes isolated small portions of tissue within the tumoral context (Figure 12). The epithelial cells in the same samples also showed cell membrane disintegration. Stromal B cells and epithelial cells within the destroyed tissue are therefore subject to similar dynamics. Previous studies have shown that metalloproteinase-2 (MMP-2) and metalloproteinase-9 (MMP-9) are produced by many white blood cells in a cell-specific manner in areas of invasive tumor growth and are associated with a greater accumulation of  $\beta$ -catenin in colon carcinomas [29,30].



**Figure 9:** Histogical context of IPCs with individual accumulation of fluorescence (Fluorescence microscopy; magnification 40x).



Figure 10: The fragments bind fluoresceinate anti- $\mu$  chain antibodies and fluoresceinate RGD and YWTD antigens (Fluorescence microscopy, detail).

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# The distribution of fluoresceinate RGD and fluoresceinate YWTD antigens coincides with that of $\mu$ chains in colorotectal adenomas and adenocarcinomas

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The histological sections were three micron deep and morphologically identical and made it possible to compare the distribution of fluoresceinate antigens. The distribution of the fluoresceinate YWTD and RGD oligopeptides was the same as that of the anti- $\mu$  chain antibodies in all of the samples, thus confirming the presence of different types of B cells associated with different levels of tissue integrity. The fluoresceinate antigens also bound the B cell fragments.

# Streptavidin binding in colorectal adenomas and adenocarcinomas

The brown staining that identified streptavidin binding, which was independent of biotin recognition, delineated cell structures with the same distribution and morphology as those of the B cells revealed by IFI (Figures 13-15). The brown monomorphic cells were recognisably



Figure 11: B cell membrane fragments are detectable as single membranous fragments.



Figure 12: Concentrated on irregular membranous structures that isolate small tissue portions within the tumoral context (Fluorescence microscopy; magnification 20x).

B cell fragments	Low-grade dysplasia tubular adenoma (n=10)	High-grade dysplasia tubular adenoma (n=12)	Adenocarcinoma (n=24)	р
None <10% of total µ chains	7 (70.0%) 3 (30.0%)	3 (25.0%) 3 (25.0%)	0	
10-40% of total μ chains >40% of total μ chains	0 0	6 (50.0%) 0	14 (58.3%) 10 (41.7%)	<0.001

 Table 5: Correlations between the percentage of B cell fragments and histological features.

IPCs with a low cytoplasmic/nuclear ratio (Figure 16) and clearly distinguishable from the monocytes and T lymphocytes stained with hematoxylin. It was also possible to identify cells with irregular nuclei (Figures 17 and 18) in which the brown staining had an intracellular



Figure 13: Streptavidin distribution (brown staining) on the sequential biopsy specimen of the same sample recalls the IFI image (Optical microscopy, magnification 20x; detail).



Figure 14: Streptavidin (brown staining) has the same distribution of fluoresceinate reagents (Optical microscopy, magnification 20x).



Figure 15: Distribution of streptavidin (brown staining) in an adenocarcinoma (Optical microscopy, magnification 20x).

granular distribution. The isolated groups of IPCs with a low cytoplasmic/nuclear ratio that showed the accumulation of fluorescence



Figure 16: Optical microscopy of histochemistry of IPCs with a low cytoplasmic/ nuclear ratio (detail).



Figure 17: B cells with irregular nuclei stained with streptavidin (detail).



Figure 18: Distribution of IPCs with irregular nuclei stained with streptavidin (brown) (Optical microscopy, detail).

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in the chain study were histochemically characterized by their irregular shapes and surfaces. They intensively bound streptavidin (Figures 19 and 20). This distribution of reagents suggested probable reactivity against the tissue component [5], but it is also possible that the matrix receptor expression of appropriately stimulated B cells differentiating *in vivo* is altered and induces dramatic morphological changes that can consist of extended filopodia or dendritic processes [31].

The B cell fragments revealed by the fluorescence studies were not clearly visible on the histochemistry slides, probably because the histochemical method is less sensitive. However, the locations of the other cell types that take up the counterstain were easily identified and revealed the considerable cellularity of the stroma and the distribution of monocytes and lymphocyte aggregates, which are often around B cell aggregates. The other cell types in the sections do not bind streptavidin.

The slides treated with streptavidin also showed that the B cells



Figure 19: The same cells are histochemically characterised by irregular shapes and surfaces (Optical microscopy, detail).



Figure 20: Streptavidin staining clearly shows the localisation of the same cells (brown) in tissue areas with loss of epithelial cell integrity (Optical microscopy; magnification 10x).



Figure 21: Streptavidin (brown staining) has the same distribution of fluoresceinate reagents (optical microscopy, magnification 20x, detail).

with a low nucleus/cytoplasmic ratio delineate small areas of tissue disruption with vascular-like aspects (Figure 21).

## Discussion

The findings of this study confirm the presence of IPCs in adenomas and adenocarcinomas and the positive correlation between immunoglobulin expression and malignancy. Direct observations of tumor tissue showed that B cells undergo morphological changes that depend on the environment in which they interact and even bind short sequences of modular oligopeptidic components involved in carcinogenesis that were artificially added to the histological samples.

Lymphocytes with a low cytoplasmic/nuclear ratio are found in ulcerative colitis [32] and associated with modified Th2 cytokines [33], whereas IPCs with irregular nuclei are prevalent in Crohn's disease [34] and associated with Th1/Th17 cytokines. In the case of tumors, this dualism recalls tumor-associated macrophages (TAMs). It has long been known that classically polarized, activated M1 macrophages are induced by IFNy alone and oriented to mediate resistance against tumors, whereas the alternative M2 form of macrophage activation is a generic name used for various forms of non-classically activated macrophages as a result of exposure to Th2. TAMs from established tumors have the properties of M2-activated cells. Many studies have investigated the connections between inflammation and cancer: Two processes that are linked through pathways that converge on transcriptom factors, cytokines and chemokines and reveal the importance of the ECM in cancer-related inflammation [35]. Recently it was demonstrated that plasmablasts was enriched in the colorectal tumor microenvironment and it was hired that these cells could be utilized in future therapeutic strategies [36]. However our findings concerning binding between Igs and modular oligopeptides underline the presence of protein-protein interactions in the stroma and it puts questions on the role of IPCs in the colorectal tumors. We show that, in spontaneous colorectal adenomas and adenocarcinomas, only µ chains bind in a detectable manner RGD, RYD mimetic RGD peptide and YWTD, thus confirming that Igs alone can create a biophysical network of the short peptidic sequences available within the ECM in this experimental context. Furthermore, the fact that Igs occur across different oligopeptidic sequences suggests that the specificity is not necessary. Biophysical protein-protein interaction studies have

shown that protease inhibitors and signaling proteins have optimized their interfaces to suit their biological functions, but antibody/antigen complexes do not show any distinctive type of interaction [37]. It has been demonstrated that in the presence of cationic components near aromatic components, the geometry is biased towards a favorable cation- $\pi$  interaction. A herringbone shape is a major configuration in transient protein-protein interfaces [38]. The contemporary presence of RGD or YWTD and B cells in the stroma in an inusual condition in the healthy tissue. However tissutal remodeling that induce the RGD and YWTD exposition can effectively occur in vivo. Felding-Habermann et al. have demonstrated that the antibodies specific for the activated conformation of the adhesion receptor integrin are part of a patient's surveillance system against metastatic cells [39,40]. However, investigations of the binding characteristics of an anti-ß1 integrin subunit antibody have shown that the epitope in the resting non-ligated integrin complex is inaccessible to it [41,42]. Biochemical studies suggest that the RGD and RGD-binding site becomes more accessible only upon receptor activation [43,44] and that the presence of the RGD motif exposed in the stroma is a strategic event. Felsenfeld et al. [45] has shown that gold particles attached to  $\beta 1$  integrin by a monoclonal antibody diffuse freely in the membrane, but the addition of soluble RGD peptide or the use of fibronectin-coated gold particles causes the attachment of integrins to the rearward-moving cytoskeleton and deletion of the  $\beta$ 1 cytoplasmic tail blocks cytoskeletal attachment [45]. Our work shows that the presence of B cells within the stroma attracts the RGD tri-peptide motif artificially added on tumoral tissue. This behavior in vivo can cause the removal of RGD/MIDAS ties in a situation of integrin activation. In pharmacology, the RGD peptide can induce tumor apoptosis in a dose-dependent manner in vitro and reduces tumor growth in vivo [16]. Accordingly to it, the presence of Igs capable to remove the RGD in the tumoral tissue configures a competition situation between RGD/Igs complex and RGD/MIDAS complex and indicates a probable B cells stimulating function in the progression of colorectal cancer.

Instead, the esposition of YWTD sequences in LRP6 is strictly dependent on the lipid metabolism and the strategic function of YWTD allows a close relationship between lipid metabolism and stromal architecture. The organization of YWTD motifs in LRP5/6 is unique among the members of the LDLR family [46] because they are the first sequences in contact with the stroma. Experimental evidence suggests that YWTD is involved in the acid-dependent dissociation of the receptor from LDL and its subsequent recycling [22] and the pH-induced changes in EGF and/or YWTD repeats must alter ligand binding properties at the amino-terminal portion of the molecule [47]. Deletion of the EGF/YWTD repeat region in the LDL receptor also generates a receptor that has a reduced capacity to bind LDL, but not  $\beta$ -VLDL and a receptor that is rapidly degraded after ligand binding [48]. Any changes that alter the conserved Asp residues are acceptors of structurally conserved hydrogen bonds and are likely to disrupt the structure of the propeller containing YWTD and prevent efficient receptor transport to the cell surface [20]. An alteration of YWTD ties means a stromal involvement because YWTD is competing with components that induce a compact stromal structure and those capable of destabilizing the cell membrane: For example, sclerostin can bind to both LRP5 and LRP6 at the level of the first two YWTD-EGF repeat domains acting as a Wnt antagonist [49]. Besides hypermorphic mutations in the WNT co-receptor LRP5 suggest that a similar YWTD  $\beta$  propeller interface is used to bind the ligands that function in developmental pathways [50]. During developmental processes, the expression of Wnts and WNT receptors are regulated temporally Page 9 of 10

and spatially and how signaling specificity is achieved in the presence of multiple ligands and receptors is a very important question [51]. In our experimental contest, the fact that YWTDs artificially added within tumoural tissue react exclusively with Igs shows that *in vivo* B cells could attend the competitive mechanism of epithelial membrane stabilization/destabilization in presence of an LRP6 degenerative process.

This work evidences that the B cells, unlike other cellular types, can interact within tumoral tissue in an aspecific manner with two polypeptides strictly connected with the cancerogenesis and reconsiders the importance of the mimetic peptides in cancer therapy. In fact, streptavidin also exclusively binds IgM in the histological context. This presupposes that the RGD-like oligopeptides studied in pathology and pharmacology do not only compete with fibronectin at the level of the fibronectin ligand binding site within integrins, but mainly compete at the level of the RGD/IgM complex. RGD-modified drugs and imaging agents have been investigated and developed by conjugating the RGD peptide with a carrier device. Our findings show that an aspecific IgM/YWTD should also be considered. Using substances capable of supporting IgM/YWTD binding competition it could stop an outside-in mechanism localized to the stroma/epithelium interface.

## Conclusion

*In vitro*, within tumoral tissue only B cells bind the modular YWTD and RGD, two polypeptides strictly connected with the cancerogenesis, by means of an aspecific protein-protein interaction. The RGD-like oligopeptides too mainly compete at the level of the RGD/IgM complex. This fact emphasises the importance of the mimetic peptides in cancer therapy and show that an aspecific IgM/YWTD should also be considered.

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