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Serological immune response against ADAM10 in Colorectal cancer patients is a favourable signature

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"Tutto l'universo cospira affinché, chi lo desidera con tutto sé stesso, possa riuscire a realizzare i propri sogni"

Paulo Coelho, *L'Alchimista*

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ABSTRACT

In cancer the immune system is activated in response to qualitative and quantitative aberrant expression by tumor cells of certain proteins named tumor-associated antigens (TAAs). In a previous study in the laboratory, has been investigated the presence of TAAs inducing auto-antibodies (auto-Abs) in the Colorectal cancer (Crc) by exploiting the patients serological reactivity against the surface membrane proteoma. Among several candidates, a specific immunoreactivity against A Disintegrin And Metalloprotease 10 (ADAM10) protein has been identify.ADAM10 is a disintegrin metalloprotease with a potential role in tumor progression and invasion due to its sheddase activity. ADAM10 is able to promote ERK1/2 signaling activation promoting cell proliferation by the cleavage of the extracellular domain (ECD) of the HER2 tyrosine kinase receptor, which is released in serum. Moreover, ADAM10 plays a critical physiological function in the epithelial morphogenesis since its sheddase activity is crucial for the cell migration and extracellular matrix (ECM) remodelling, a phenomenon that also drives tumor metastasis. Thus, an aberrant expression of the ADAM10 in epithelial tissues might elicit a specific serological immune response. The serological screening performed on purified ADAM10, showed a significant presence of immunoreactivity against ADAM10 in the sera from patients affected by tumors of epithelial origin like Crc, Pancreatic cancer (Pc) and Breast cancer (Brc) if compared with serological reactivity of healthy subjects. On the contrary, sera from patients affected by hematological malignancies such as chronic lymphocytic leukemia (B-CLL) and Multiple Myeloma (MM), did not showed significant immunoreactivity against ADAM10. These results suggested that the presence of auto-Abs against ADAM10 in patients sera might be a candidate biomarker for carcinomas of epithelial origin. Since it is known that ADAM10 is overexpressed in advanced stages of the Crc disease, when the lymph nodes are infiltrated

by the tumor, we evaluated the disease course of this patients after surgical resection in order to define whether the presence of auto-Abs anti-ADAM10 is a favourable or detrimental signature, correlating the immunoreactivity with the patients follow-up. The results showed that the presence of auto-Abs anti-ADAM10 prolonged significantly the disease-free condition if compared with Crc patients without serological immunoreactivity against ADAM10. Thus, in order to investigate the effects of these auto-Abs, *in vitro* experiments of both HER2 ECD release and cell migration were performed using the Colon carcinoma cell line LoVo. Results showed that commercial anti-ADAM10 antibodies resulted to be effective in inhibiting cell migration and HER2 ECD release in LoVo cell line while IgG fraction purified from representative Crc patients did not. This lack of inhibition might be the consequence of the fact that LoVo cell line did not express the specific epitope that elicited the serological reactivity in Crc patients. In fact, Crc patients showed different expression of ADAM10 in tumoral tissues. Patients with a positive immunoreactivity against ADAM10 (Ser-ADAM10 +) showed an overexpression of the inactive form of ADAM10 and, accordingly, an high expression of the inactive HER2 (p195) isoform and a decreased expression of phosphorilated-ERK1/2. These results suggest a reduced activity of ADAM10 on the HER2 ECD cleaveage which is supported by the observation that Ser-ADAM10 + patients showed lower HER2 ECD concentrations in serum compared to Ser-ADAM10 - patients.

In conclusion, the presence of auto-antibodies anti-ADAM10 may be considered a biomarker of favourable prognosis for Crc patients at advanced stages of the disease that reflects an overexpression of inactive ADAM10 during the cell transformation and accordingly the lower protein maturation leads to a decrease of its sheddase activity which in turnt declines the proliferative and invasive capacity of tumor cells.

1. INTRODUCTION

1.1. Etiology of Cancer.

According to the generally accepted monoclonal theory, cancer is a genetic disorder which originates from a single cell, after the addition of multiple mutations in certain genes that control cell behavior, which in turn promote invasion of other tissues and uncontrolled multiplication [1].

The genetic alterations that cause cancer may act on three different types of genes producing opposite effects [2]:

1. *Tumor suppresor Genes*: genes whose function is to limit cell division and remove dead or damaged cells, such genes may be inactivated.
2. *Protooncogenes*: genes whose function is to promote movement and proliferation in normal cells, these genes may be abnormally activated. Protooncogenes can be converted in oncogene due to a mutation and, subsequently, oncogenes are translated into oncoproteins with abnormal enhancement of activity.
3. *DNA repairing genes*: genes that are involve in DNA repair and that may be inactivated.

The result is the inactivation of tumor suppresor genes or activation of oncogenes. The classical theory of carcinogenesis is summarized in three consecutive phases known as initation, promotion and progression **(Figure 1).** Initiation phase comprising the time in which carcinogenesis initiators such as UV light, ionization radiation, thermal disruption, or chemical sources induce an irreversible event in a single cell and the DNA damage remains unrepaired [3]. In the promotion phase , the damaged cell plays a tumor promoter role, they can be inducted to proliferate and undergo clonal expansion in which a generation of cells with mutated genes is formed. The progression of the tumor take place when further mutations, epigenetic changes or genetic instability confer to the cells a more aggressive capacity to invade distant places, leading to malignant tumour transformation.

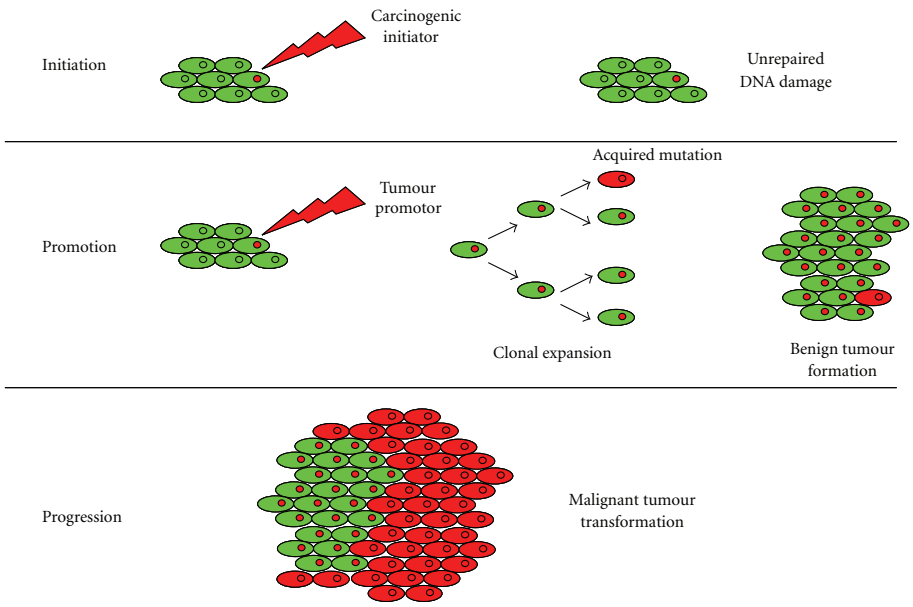


Figure 1. Monoclonal theory of carcinogenesis. [From 2]

1.2. Colorectal cancer (Crc).

Colorectal cancer is one of the leading causes of lethality related to tumor pathologies in the United States, Crc is the third most common cancer both in men and women. Despite conventional treatments (surgery, chemoterapy, and radiotherapy) for Colorectal cancer which have improved in recent years, individuals with advanced disease still have a poor prognosis [4].

The Crc survival rate is calculated according to the percentage of people who survive at least five years after the cancer diagnosis, excluding those individuals that die by other unrelated causes.

The five-years survival rate depends on the clinical development of the disease at the first hospitalization:

- Is 90% in cancer in the early stage (in situ).
- Is 70% in cancer cells are found nearby lymph nodes or organs.
- Is 13% when cancer cells are found in distant parts of the body.

Nevertheless, surgical removal of the tumor supported by chemoterapy and radiotherapy treatments may improve the five-years survival rate for the patients.

1.2.1. Colon and rectum: General description.

The last part of the digestive system is the large intestine which is consecutive to the small intestine (ileum) and, from there to the final is composed by cecum, ascending colon, transverse colon, descending colon, sigmoid colon, rectum and anus (**Figure 2**).

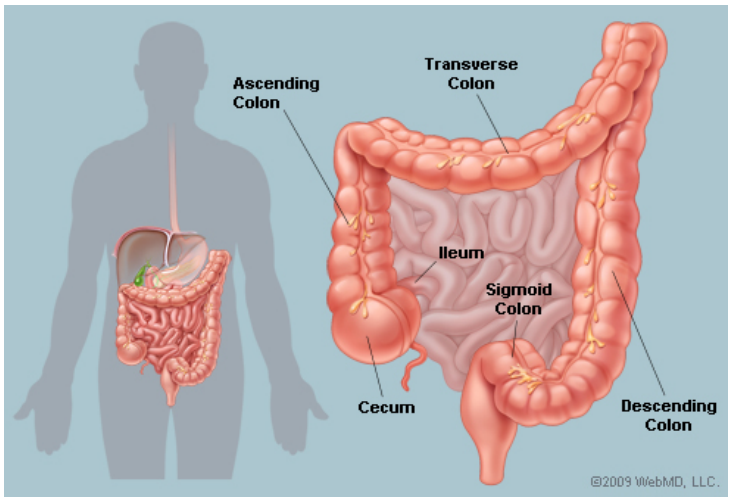


Figure 2. Colon and rectum anatomy [From 5].

The colon is the part of the large intestine with the function of absorbing water, salts and some vitamins from solid waste before they are discarded. Moreover the colon mantains the fluid balance and, with the aid of the gut flora, is able to ferment the undigested carbohydrates, favouring the subsequent absortion of these products by the cells of the intestinal lining. After the fermentation and absorption, the remaining waste products, named feces, reach the rectum and are discarded through the anus.

The colon has an inside space called **lumen** and histologically from the inner lining towards the outside is made up by several layers **(Figure 3)**:

. The Mucosa, the innermost part of which is the glandular *epithelium* forming the villi which project into the intestinal tract and the characteristics crypts of Lieberkühn that project into the deeper part of the mucosae; subsequentely there is the *lamina propria* containing reticular connective tissue and mucosal glands, and finally the *muscularis mucosae* that separates mucosa and submucosa.

. The Submucosa, is composed by dense irregular connetive tissue that contain blood vessels, lymphatic vessels, nerve fibers and mucosal glands**.**

. The Muscularis, composed by two consecutive layers of smooth muscle, gives to the intestine the ability to contract. The inner layer is *circular* while the outer layer is *longitudinal*. Between the two muscle layers there is the myenteric or Auerbach's plexus which controls the coordinated contractions of these layers.

. The Serosa, is a smooth membrane which comprises two layers. The outer layer consists of secretory *epithelial cells*, and the inner layer consists of *connective tissue*.

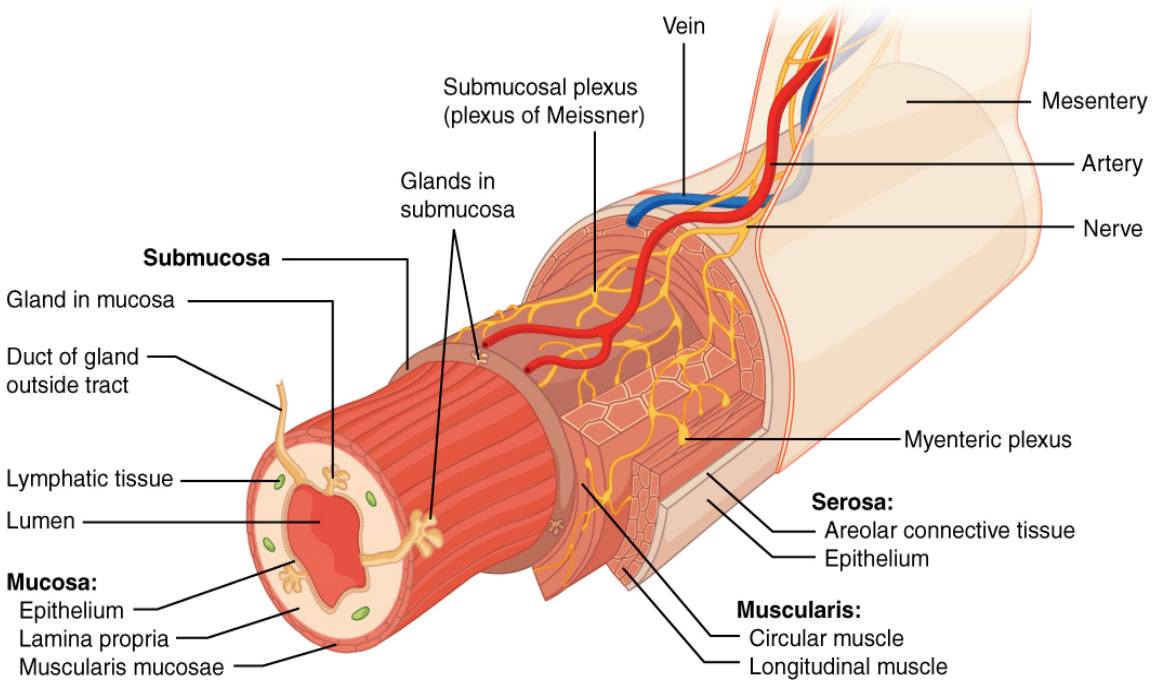


Figure 3. Schematic image representing the several layers of the colon [From 6].

1.2.2. Colorectal cancer development: adenoma-carcinoma sequence.

There are several types of Crc, the adenocarcionoma is the most frequent with an incidence of 90-95% of all Colorectal cancers affecting the glandular epithelium, specifically the lining of colon and rectum. Cells develop an ordered series of events beginning with the transformation of normal colonic epithelium to an adenomatous intermediate and subsequently to an adenocarcinoma (**Figure 4**).

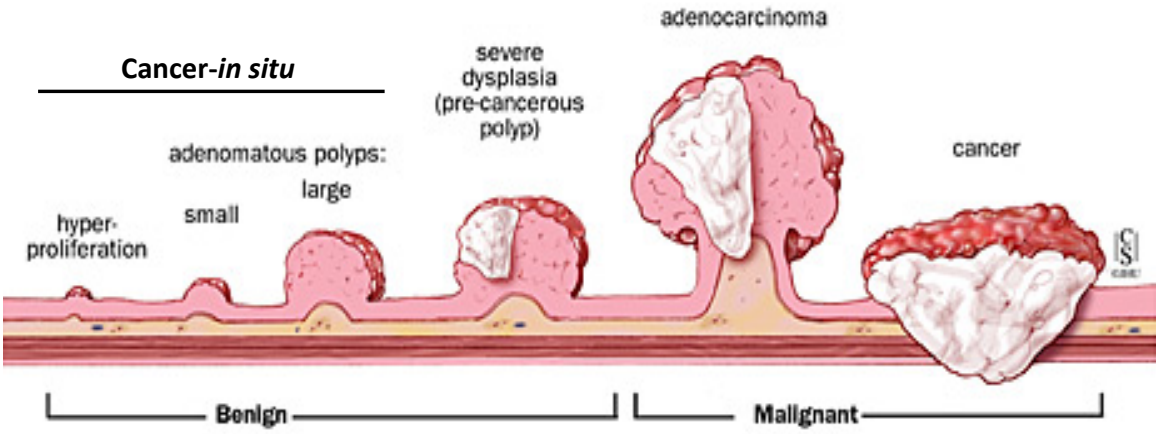


Figure 4. Progression of tumoral mass through the colon layers. The adenocarcinoma starts from the glandular epithelium where it forms polyps which can evolve in adenomas. The adenomas are polyps with different microscopic aspect that show an abnormal cell growth, and when are limitated to the mucosa, they are known as cancer-*in situ*. The polyps are kept in the area where they first developed and are not able to spread. When polyps became able to reach the submucosa, are considered adenocarcinomas and therefore malignant.

There is no particular cause that trigger the disease, except for the development of **polyps**. These projecting mass of overgrown tissue from the inner lining to the lumen would be a clonal expansion of the tumor promoter according with the Monoclonal theory of carcinogenesis [1] (**Figure 1**) and might develop in cancer as consequence of new mutation events that would contribute to tumor expansion. The polyps are considered benign unless an abnormal cell growth is detected microscopically; in this case the growing cells are

defined **adenoma** and are limited to the inner layer being not able to spread beyond of the mucosa. The polyps and adenomas are also defined *cancer-in-situ*. When the tumor reaches the submucosa, it has the potential to spread and is defined as **invasive adenocarcinoma**, a malignant epithelial tumor that is capable of growth exceeding the different layers of the colon as its size increases dimensions.

When the primary tumor has gone through all strata, it may leave the epithelium and **infiltrate nearby lymph nodes** as well as it may reach other parts of the body giving rise to distant metastasis.

1.2.3. Stages in Colorectal cancer.

Crc are classified according to their pathological stages, based on the size and invasiveness of the tumor. The most commonly used staging system of classification which is accepted by the American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC) is based on Tumor/Nodes/Metastasis (**TNM**) score which define important pathological transition phases (**Figure 5**).

The three letters combined in AJCC system mean the following: **T** determines the size of the primary tumor; **N** informs about the lymph nodes infiltration both near and distant lymph nodes; while **M** defines the presence of metastasis and the possible formation of secondary tumors by spreading of primary tumor:

* **Primary Tumor (T)**.

TX: Primary tumor cannot be evaluated.

T0: No evidence of primary tumor.

Tis: Carcinoma *in situ* (CIS; abnormal cells are present but have not spread to neighboring tissue; although is not a cancer, CIS may become cancer and is sometimes called preinvasive cancer).

T1, T2, T3, T4: Size and/or extent of the primary tumor.

* **Regional Lymph Nodes (N).**

NX: Regional lymph nodes cannot be evaluated.

N0: No regional lymph node involvement.

N1, N2, N3: Degree of regional lymph node involvement (number and location of lymph nodes).

* **Distant Metastasis (M)**.

MX: Distant metastasis cannot be evaluated.

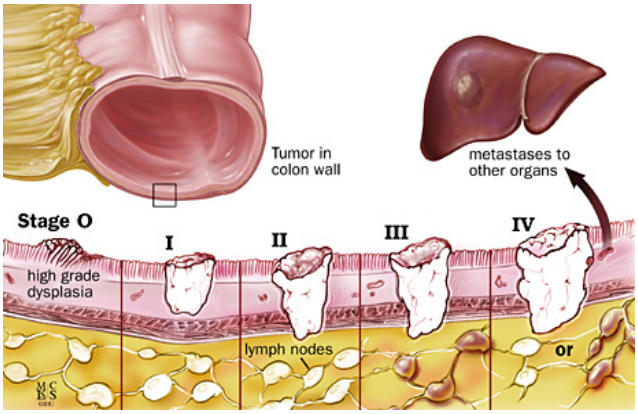
M0: No distant metastasis.

M1: Distant metastasis are present.

TNM Classification (American Joint Commission on Cancer)

Stages	T Main tumor	N Lymph nodes	M Metastatic disease
Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
	T2	N0	M0
Stage II	T3	N0	M0
	T4	N0	M0
Stage III	Any T	N1	M0
	Any T	N2	M0
Stage IV	Any T	Any N	M1

(A)



(B)

Figure 5. Tumor/Nodes/Metastasis (TNM) classification in Colon cancer **(A)** and schematic representation of different stages **(B).** Depending on the mass tumor invasion of different layers of the colon, an internal stadium classification T that measure the extent of the primary tumor is assigned. The presence or absence of infiltrate lymph nodes is indicated by the designation N, while M reports signs of metastasis and spread to other organs [From 7].

1.3. Antibody-mediated response against Tumor Associated Antigens in cancer.

The immune system is able to distinguish between self and non-self antigens in order to protect our body against potentially damaging foreign organisms that may cause disease. The non-self proteins bear special tags that are recognized by our immune system triggering an adaptive response mediated by both T cell (cell mediated immune response) and B cell (antibodies immune response). On the contrary the self proteins do not carry any label and are ignored by the immune system with the exception of autoimmune diseases. It is well known that in cancer, the self-tolerance is broken in an effort to eliminate malignant cells and the immune system is activated in response to aberrant qualitative and quantitative expression by tumor cells of certain proteins named tumor-associated antigens (TAAs) [8, 9].

TAAs are classified into several categories on the basis of their expression characteristics [10]:

Mutated — these are proteins with unique mutations in their amino acid sequence that are present only in cancer cells.

Differentiation — expressed by a limited range of normal tissues.

Overexpressed — these antigens can be expressed on a wide range of normal tissues, but are expressed at much more high levels in tumours.

Idiotypic — unique protein sequences in the T cell receptor (TCR) or B cell receptor of leukaemias and lymphomas.

Oncoviral — proteins encoded by tumorigenic viruses.

These aberrant tumor-associated proteins may confer an advantage to the tumor cells both in uncontrolled growth and invasion capability [2]. The humoral immune response is induced to produce auto-Abs anti-TAAs with the support of T-helper cells, with the aim to

recognize the aberrant proteins as foreign antigens. The presence in the serum of auto-Abs anti-TAAs reflects the occurrance of molecular events related to malignant transformation due to dysregulation of cellular mechanism.

At present, it is not known the mechanism by which the TAAs become immunogenic, however the recognition of self-proteins by the immune system may be leaded by changes in the TAAs structure, their aberrant regulation or their overexpression [11]. It has been reported that most of the target antigens are cellular proteins with possible functions in tumorigenesis pathways such as tumor-suppressor p53 [12, 13] or proteins involved in cell progression and invasion such as HER2/neu [14]. The modifications of these proteins might also alter their normal physiological function and the protein dysregulation could contribute to cancer development [2].

1.4. Immunoproteomics.

Immunoproteomics is a methodological approach that exploit the presence of auto-Abs in the sera of patients for the identification of antigenic proteins eliciting the humoral response in different pathologies such as autoimmune disease, infection and cancer [15, 16, 17].

The immunoroteomics approach exploit the fact that auto-Abs in the serum are stable with a long half-life and that the amplified humoral response make easier the detection also of antigens that are poorly expressed.

The immunoproteome-based approach is one of the methodologies largely used for the identification and characterization of new TAAs in cancer. Over the past decade, the proteomic techniques have experimented a technological development that has allowed to screen a large member of sera in order to improve the detection of many TAAs concomitantly [18, 19].

Serological proteome analysis (SERPA) uses patient sera to screen proteins from primary tumor tissues or cell lines combining the separation of tumor proteins on two-dimensional gel electrophresis (2DE), Western blot with patients and healthy subjects sera, image analysis and antigenic protein identification by mass spectrometry (MS) analysis (**Figure 6**). This approach allows the screening of a large number of patient sera and to establish the frequency of the reactivity against relevant auto-antigens [20]. The high throughput screening can be done on available tumor cell lines being reported that the serological reactivity on cell lines are comparable to that obtained on the autologous tumor proteome (**Figure 7**) [19]. In addition, SERPA enables to distinguish immunoreactivity toward protein isoforms and/or directed against post-translational modifications (PTM) (such as phosphorylation, glycosylation or protein cleavage), which is of central importance, being the protein immunogenicity frequently associated to unusual isoforms or to different PTMs occurring during tumoral transformation [19, 21].

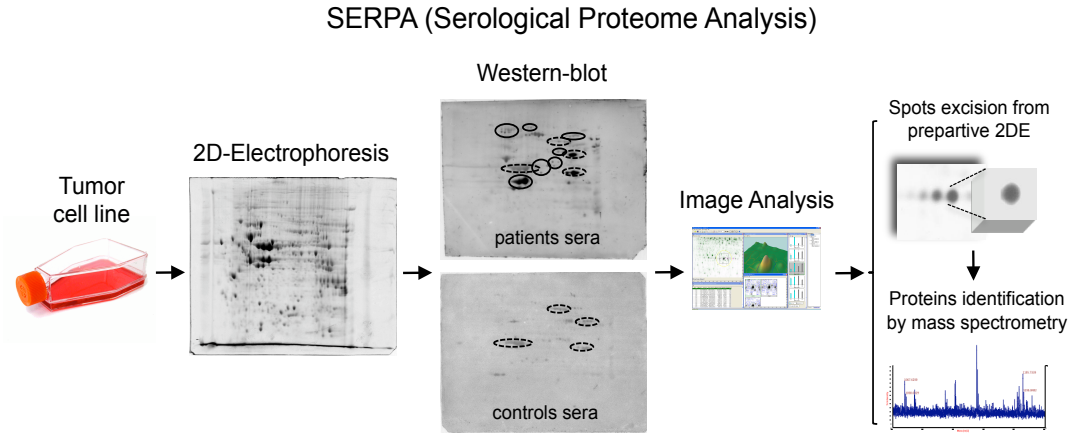


Figure 6. Schematic representation of the workflow for tumor associated antigens identification by SERPA that exploits the presence of autoantibodies directed against tumoral proteins in the sera of patients.

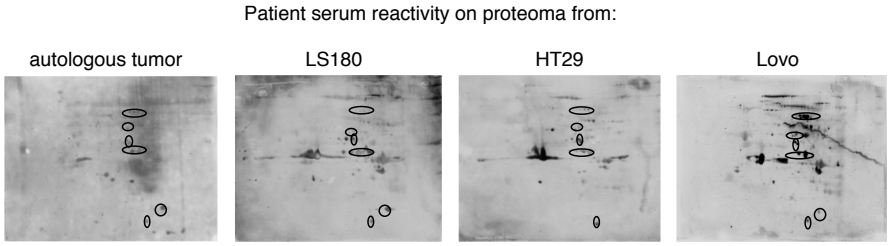


Figure. 7 Proteome obtained from tumor cell lines is representative of the primary tumor proteome. Colon cancer patient serum reactivity on the autologous tumor proteome and on the proteome from three different Colon cancer cell lines (LS180, HT29 and LoVo) shows shared reactivities (underlined by circles).

1.4.1. Immunoproteomics on Colorectal cancer.

In our laboratory, a SERPA on the proteome of the LS180 Colon carcinoma cell line has been performed using sera from Crc patients with the aim of identify news TAAs. A panel of six intracellular proteins eliciting a serological immune response in Crc patients has been identified [19]. However, none of these proteins specifically expressed or over-expressed in tumor cells, that elicited an humoral response in Crc, were surface membrane expressed proteins. Since the surface membrane proteins could represent an ideal therapeutic target in cancer, in order to identify auto-Abs directed against putative TAAs in the surface membrane compartment, an enrichment procedure of this compartment was performed using surface protein biotinylation followed by affinity chromatography. The enriched material was coupled with SERPA performed using pool of sera from Crc patients and a pool of sera from healthy subjects.

This approach allowed the identification of some candidate proteins as TAAs, among these the membrane metalloprotease ADAM10 (A Disintegrin And Metalloprotease 10) [23]. ADAM10 is a cell surface metalloproteinasa that plays a potencial role in tumor progression and invasion [22]. The ADAM10 protein spot resolved by 2DE was specifically recognized by the pool of Crc patients sera, while the pool of Cn sera did not recognized it

(**Figure 8A**). The same spot was reactive with the avidin-HRP as confirmation of its surface expression and was recognized by commercial antibody specific for ADAM10 (**Figure 8A**) confirming the mass spectrometry (MS) identification. The surface expression of ADAM10 on LS180 cell line has been confirmed by immunofluorescence, being the reactivity of the anti-ADAM10 antibody present both in permeabilized and non-permeabilzed cells; the reactivity of the surface HLA class I molecule, and the reactivity against the intracellular protein β-actin, have been used as controls (**Figure 8B**).

Table 1. Protein identification by MALDI-TOF MS analysis biotin-labelled spot [From 23].

Spot	IPI	Acc.No. Swissprot	Entry name	Protein name	Seq. Cov.	*pI* / Mr exp	Mowse Score	Meas. Pept.	Matc. Pep.
1	IPI00013897	O14672	ADA_10HUMAN	ADAM 10	16%	5.59 / 66346	90	16	10

IPI: International Protein Index; Acc. No.: Accession number; Seq. Cov.: sequence coverage; *pI*: isoelectric point; Mr: relative molecular mass; exp.: experimental; MOWSE: probability based score used by Mascot programme; Meas. Pep.: measured peptides; Matc. Pep.: matching peptides; WB: validation by Western blot

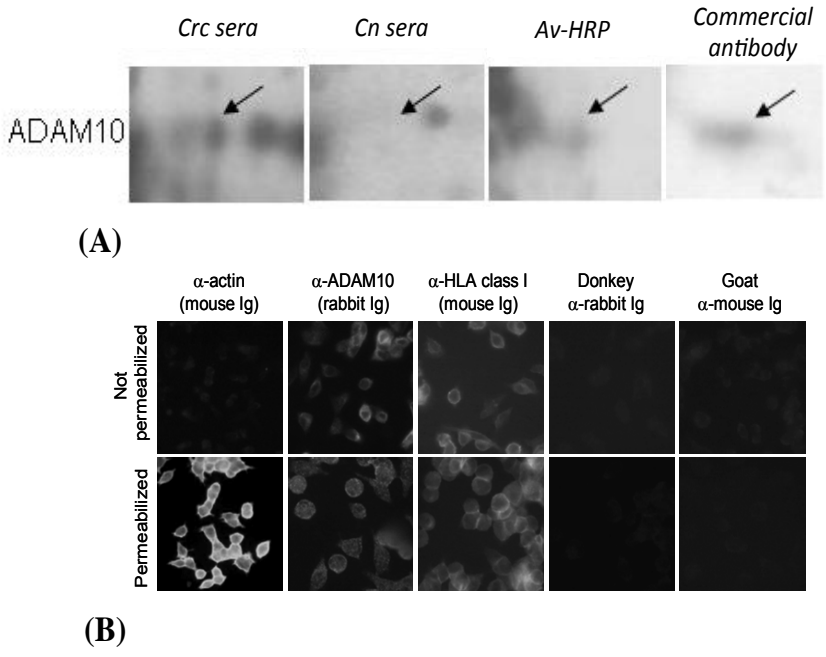


Figure 8. (A) Serological Reactivity against ADAM10 enriched by cell surface biotinylation. Reactivity was obtained using a pool of Crc sera, a pool of Cn sera, avidin-HRP and commercial anti-ADAM10 antibody. **(B)** Immunofluorescence analysis on both permeabilized and non-permeabilized LS180 cells [From 23].

1.5. General description of ADAM10.

ADAM10, also named Kuzbanian, is a surface type I transmembrane glycoprotein of 748 amino acids in length with a conserved domain organization [24]. From the extracellular to intracellular domain, ADAM10 is composed by a Prodomain (P), Metalloproteinase (M), a Disintegrin-like (D), a cystein-rich (Cys), a transmembrane (T) and a cytoplasmic (C) domains [25, 26, 27].

The prodomain contains a signal peptide that permit an efficient transport of the protein to the membrane and contributes to the correct protein folding because acts as a molecular chaperone. In addition, the prodomain maintains ADAM10 in an inactive form preventing enzymatic activities when not required, in fact ADAM10 is a proteinase enzyme initially synthesized as inactive precursor protein of 98 kDa [28]. After synthesis and translocation in the endoplasmic reticulum ADAM10 is delivered and further processed to mature form in the Golgi compartment where the protein is subjected to glycosilation. Upon transit through the trans-Golgi network, the prodomain of ADAM10 is removed by endoproteolytic processing due to activation of proprotein convertases such us PC7 or Furin [28]. Generating and enzymatically active ADAM10 of 64 kDa, the mature enzyme is then transported to the plasma membrane, where it can act in shedding the ectodomain of several different membrane bound receptors, adhesion molecules, growth factors and cytokines like TNF-alpha [29, 30, 31, 32], Notch [33, 34], E-cadherin [35], Ephrin [36], HER-2 [37], CD30 [38] and CD44 [39] receptor.

The catalytic activity of ADAM10 resides in the metalloprotease domain which contains a zinc-binding motif implicated in the hydrolytic processing of substrate proteins. The prodomain has a single cysteine residue that interacts with the zinc atom in the catalytic domain forming an intramolecular binding complex that keeps the protein in an inactive state by an inhibitory mechanism called “the cysteine switch” [40]. Pro-protein convertases

are able to remove the prodomain allowing the dissociation of the cysteine from the zinc binding site which in turn allows the exposure of the catalitic site leading the activation of the protein [28] (**Figure 9**).

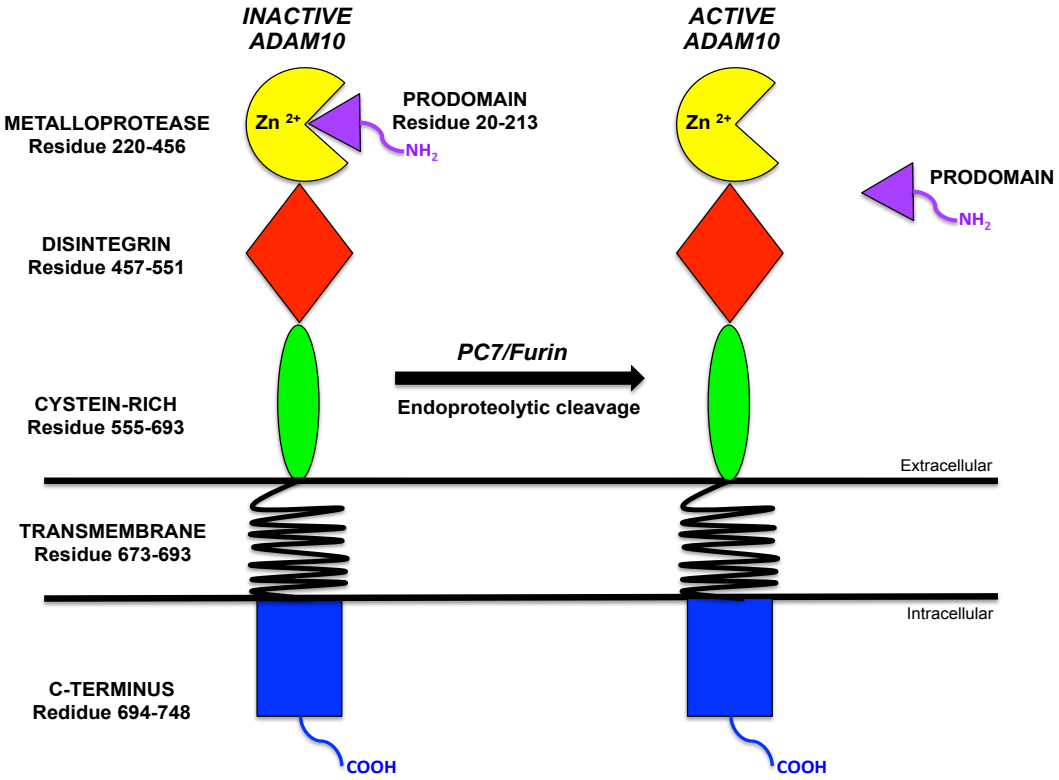


Figure 9. Schematic representation of the domains structure of ADAM10 and its activation by prodomain-cleavage.

Shedding defines a process by which several cell surface proteins are cleaved in a site close to the membrane surface, resulting in the release of the extracellular domain. This process is commonly known as Proteolytic ectodomain release [41, 42]. ADAM10 belongs to the ADAMs family which is considered as the major family of mammalian surface membrane proteins with ectodomain sheddase activity [27] (**Figure 10**).

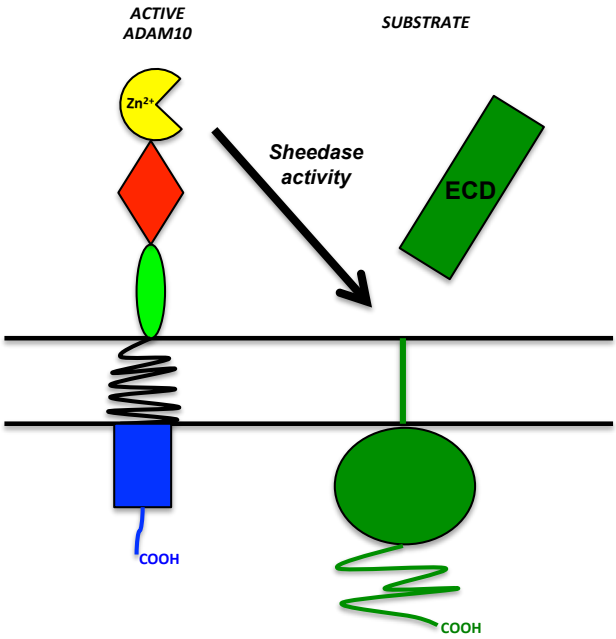


Figure 10. Schematic representation ADAM10-mediated shedding of transmembrane proteins which in turn leads to the release of soluble extracellular domains (ECD).

1.6. Roles of ADAM10 for health and disease.

ADAM10 is especially prominent in epithelial tissues such as epidermis, kidney tubules and the endocardium and myocardium of the heart. Moreover, ADAM10 is present in mesenchymal cells which have a critical role in the epithelial-to-mesenchymal transformation [43]. In particular, the function of ADAM10 is physiologically involved in ectodomain shedding of several receptors (e.g., Notch, amyloid precursor protein) [44], adhesion molecules (e.g., N-cadherin, E-cadherin) [45], and ECM components (e.g., type-IV and type XVII collagen) [46, 47] that control many processes during the development, postnatal neurogenesis, tissue morphogenesis and homeostasis [33].

In a non pathological environment, cells are exposed to physiological replacement due to cellular repair and regeneration. Cell communication is fundamental to allow cell adhesion and migration whose are key processes to perform tissue regeneration. Cells interact with their microenvironment and respond to extracellular stimuli in order to keep

under control different processes such us differentiation, proliferation, migration and death. All these different signals are transmitted to the cell through intracellular pathways and the cell has to respond based on them. Hence, any aberrant modification of these signaling pathways could contribute to the development of cancer. Moreover, cell migration is necessary to the tissue remodelling but also drives tumor metastasis during malignant transformation so it is critical for tumor cells traveling to distant sites [60].

Sheddase activity confers to ADAM10 the ability to *influence cell adhesion and cell-cell interactions* inducing the remodeling of the extracellular matrix (ECM) [61] and the ability to *regulates the activation of growth-stimulating* factors because is involved in their ectodomain-shedding promoting cell proliferation and cell survival. In particular, it has been reported that ADAM10 is the major metalloproteinase able to activate the specific cell surface receptor HER2 by shedding its ECD in breast cancer cells [37]. Since activation of HER2 participate in the regulation of important cellular functions, the ADAM10-mediated cleavage fosters cell differetiation and proliferation allowing migration and cell survival.

The dysregulation of the ADAM10 functions may result in further pathological processes including inflammation and cancer progression [49]. Indeed, the loss of the cell adhesion molecule E-cadherin was shown to promote invasiveness of tumors [50, 51]. Cell dissociation is key step to induce cell migration, invasion and consequently to promote metastasis. Interestingly, a large number of publications showed the overexpression of ADAM10 in variety of tumors: neuroblastoma, pheochromocytoma [52], hematological malignancies [53], androgen-dependent prostate cancer [55], oral squamous cell carcinoma [56], neoplastic gastric mucosa [57], uterine and ovarian carcinomas [58], and colorectal cancer [59]. In the latest, high expression of ADAM10 correlates with advanced stage of the disease [22] suggesting that this protein play an important role in tumor progression and invasion.

Aberrant qualitative or quantitative expression of ADAM10 by tumor cells might confer an advantage on tumor dissemination. Hence, the role of ADAM10 in cancer development seems to be crucial.

1.6.1. Sheddase activity on Epidermal growth factor receptor 2 (HER2).

The Epidermal Growth Factor Receptor (EGFR) family is made up by four receptor tyrosine kinases (RTK): EGFR/HER1/erbB1, HER2/erbB2, HER3/erbB3 and HER4/erbB4. HER1, HER3 and HER4 bind a soluble ligand (e.g., EGF, betacellulin, amphiregulin) allowing to change its conformation from close to open. The open conformation promotes the receptor dimerization between family members. The heterodimer formation is fundamental for the receptor activation by phosphorylation and activation of the intracellular tirosine kinase domain located at the C-terminus.

On the contrary of HER1-3, HER2 as full-length (p195) does not bind any ligand as it is constitutively in open conformation and, maybe for that reason is the preferred partner for heterodimer formation. HER2 contains an extracellular domain (divided in 4 sub-domains), a transmembrane domain, and an intracellular domain. While the intracellular domain is responsible for phosphorylation and nessesary for the signaling trasmission, the extracellular domain is the portion of the molecule where the signal transduction starts. Indeed, HER2 is activated by cleveage of its extracellular domain (ECD; p100), which is released in the extracellular space, and the truncated form (p95), which remains in the membrane shows an increased phosphorylation of the intracellular tyrosine kinase activity [63] resulting both in increased autophosphorylation and increased phosphorylation of targets molecules.

These phosphorylated residues are recognized by different downstream mediators of signal transduction that regulate several genes that affect some cellular processes such as

proliferation, migration, and cell survival [64, 65]. In particular, oncogenic signaling by HER2 involve the activation of MAPK-ERK pathway which contributes to enhanced cell division [66] (**Figure 11**). Thus, HER2 activation plays an important role in tumor invasiveness and metastasis formation.

Interestingly, ADAM10 is the major protease responsible for HER2 ectodomain sheddase activity [37] which in turn promotes HER2 activation and, accordingly, tumor progression.

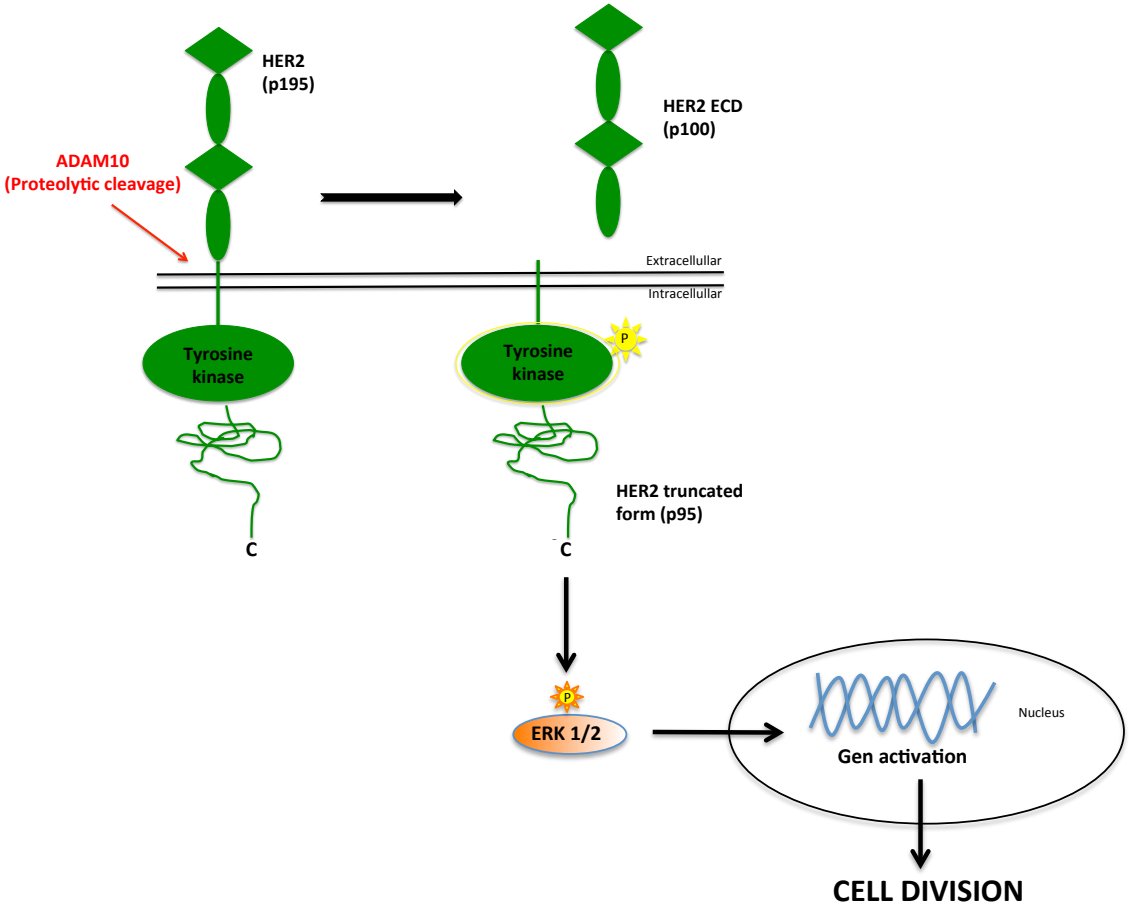


Figure 11. ADAM10 activates the HER2 tyrosine kinase receptor by ectodomain cleavage which trigger the activation of downtsream signaling pathway promoting cell division and cell survival.

2. AIM OF THE THESIS

In a previous work, ADAM10 had been identified as putative TAA eliciting a serological immune response in Crc patients. Therefore, the first aim of this project was to confirm in a larger cohort of Crc patients the presence of auto-Abs against ADAM10 and substantiate the immunogenicity of ADAM10 as TAA. In particular, we would like to investigate whether the immunoreactivity against ADAM10 correlates with the disease stages and progression.

Since ADAM10 plays an important physiological role in epithelial morphogenesis, with the aim to understand whether the immunoreactivity against a specific TAA is a feature of a specific type of tumor or is a characteristic that can be shared with others tumors, we investigated the presence of the anti-ADAM10 immunoreactivity in sera from tumors with epithelial and non-epithelial origin.

Finally, TAAs are used as early detection markers for malignant transformation, but little is known about the effects that the presence of anti-TAA auto-Abs might have on the progression of the disease. Thus, a further scope of this work was to understand whether the presence of these auto-Abs anti-ADAM10 might affect the Crc progression, either in favourable or detrimental way, and based on the results to investigate the possible effects of the auto-Abs on the physiological activity of the ADAM10.

3. RESULTS

3.1. Colorectal cancer serological screening on purified ADAM10.

With the aim to investigate ADAM10 as TAA candidate in Crc, a serological screening on purified recombinant ADAM10 (rhADAM10) resolved by SDS-PAGE was performed by Western blot. Sera from Crc patients and healthy subjects were used to test the presence of auto-Abs specific for ADAM10 as immune response elicited by the protein (**Figure 12**).

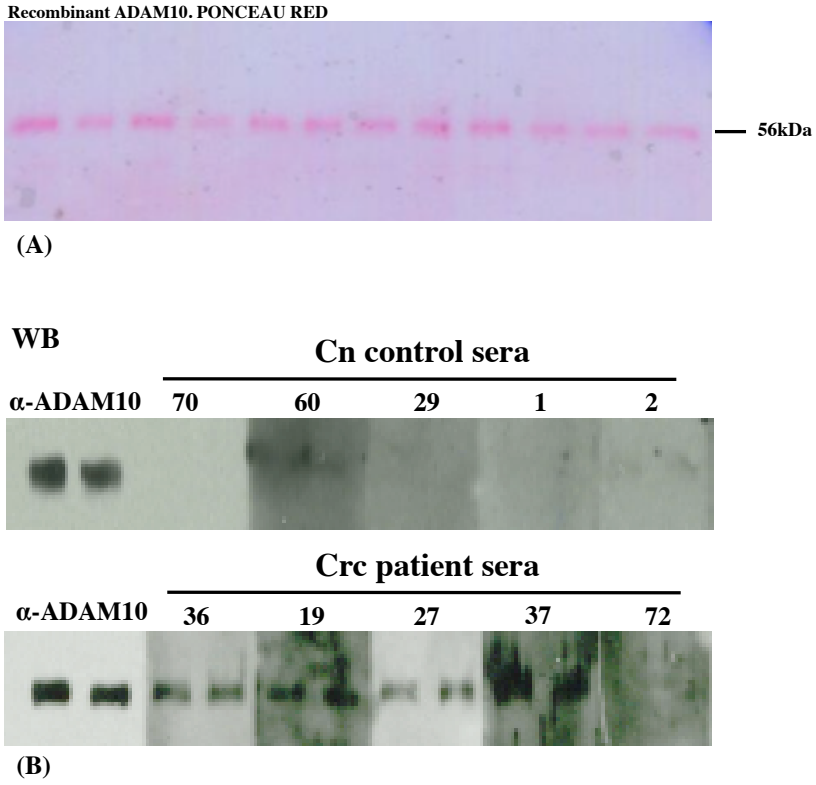


Figure 12. (A) Ponceau red staining obtained loading 200ng of rhADAM10 in each line. **(B)** Some examples of immunoreactivity obtained in the serological screening on purified rhADAM10. Control sera 70, 60, 29, 1, 2 immunoreactivity; Crc patient sera 36, 19, 27, 37, 72 immunoreactivity.

All signals were normalized according to the reactivity obtained with a commercial anti-ADAM10 antibody (R&D systems) used in all experiments. The normalized optical density (OD) was directly proportional to the intensity of immunorectivity against ADAM10 and therefore representative of the presence of auto-Abs.

Sera from 106 Crc patients were analyzed. This Crc cohort included 52 patients of stage I-II, in which the lymphonodes were not infiltrate by tumoral cells (indicated by TNM international classification as No), and 54 patients of stage III-IV, in which lymphnodes were infiltrated by cancer cells (indicated as N or M) (**Table 2**). This patient stratification is an important predictive value from the clinical point of view for the patient prognosis. As control cohort (Cn) were used 91 sera from healthy subjects or subjects with pathologies not-correlated with tumoral transformation, in order to exclude non tumor-specific immunoreactivity of these autoantibodies, like for example as induced by inflammatory status. Age and gender distribution between patients and controls groups resulted to be homogeneous with exception of comparison with breast carcinoma population (**Table 2**).

The analysis of normalized OD (OD values for each serum are reported in *Appendix*) showed that the immunoreactivity against ADAM10 was higher in patient sera compared to control sera (Cn *vs*. Crc, p<0,0001, Mann-Whitney test) (**Table 2** and **Figure 13A**). The significant difference was maintained comparing controls with either patients at early disease stages (I-II) (Cn *vs*. CrcN0, p=0,0004), and patients at late disease stages (III-IV) (Cn *vs*. CrcN, p<0,0001). Relevant differences were also found when the three independent groups were evaluated together by covariance Kruskal-Wallis test (p<0,0001) (**Figure 13A**), with post analysis test significant for Crc *vs*. Cn (p< 0,001) and CrcN *vs*. Cn (p< 0,001) comparisons.

Table 2. Serological reactivity for ADAM10 using 237.2 OD as cut-off value.

			anti-ADAM 10 reactivity		Stage I-II	anti-ADAM 10 reactivity		Stage III-IV	anti-ADAM 10 reactivity	
	n (m/f)	Age	normalized OD	237.2 OD cut-off	n (m/f)	normalized OD	237.2 OD cut-off	n (m/f)	normalized OD	237.2 OD cut-off
Crc	106 (58/48)	67.1 sd 11.6	595.8 se 56.9	78+ 28-	52 (27/25)	560.6 se 77.47	36+ 16-	54 (31/23)	629.7 se 83.57	42+ 12-
Cn	91 (37/54)	63.8 sd 14.41	261.7 se 22.26	34+ 57-	-	-	-	-	-	-

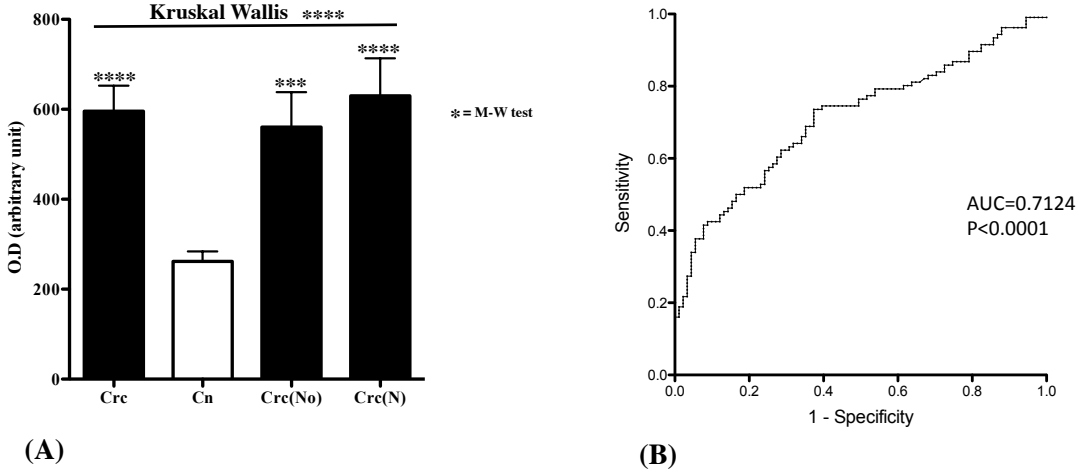


Figure 13. Analysis of the serological immunoreactivity against ADAM10 measured as Western blot signal optical density (OD). **(A)** Statistical analysis performed by Mann-Whitney (M-W) and Kruskal Wallis showed that Crc sera reactivity vs. Cn sera was extremely statistically significant on both Cn vs. Crc-N (patients at late stages III-IV) and Cn vs. Crc-No (patients at early stages I-II). **(B)** Receiver operating characteristic (ROC) analysis. Increasing sensitivity and specificity values from 0 to 1 determined for the serological screening an area under the curve (AUC) value of 0.7124 with p<0.0001 statistical significant, indicating that the serological screening on ADAM10 have a powerful discriminative capacity between the tumoral patients and control groups (**** = p<0,0001; *** = p<0,001; ** = p<0,01; * = p<0,05).

The ROC analysis of Cn *vs*. Crc anti-ADAM 10 reactivity, indicated an area under the curve (AUC) of 0.7124 with a p value <0.0001 (**Figure 13B**), which in turn suggested that the test may have a discriminating capacity between the two groups. ROC analysis was performed in order to choose the value with the best balance between sensitivity and specificity, indicating to the OD threshold value of 237.2 as discriminat in order to define the reactivity as positive (+) or negative (-). This value roughly coincide with the OD mean

value of control sera (261.7 se 22.26) and gave rise the better sensitivity of 0.74 and specificity of 0.63. The relevance of the use of this threshold value was confirmed by the Fisher's test analysis that indicated a significant discrimination between Cn and Crc patients ($p<0.0001$). When the patients were considered in two groups based on the infiltration of the lymph nodes, the Fisher's test showed higher difference in patients at later stages (III-IV) ($p<0.0001$) if compare with the group of patients at early stages (I-II) ($p=0.0003$). Sensitivity and specificity of this putative marker were confirmed for the cancer cohort *in toto* and, ss expected, the sensitivity and specificity increased if only patients at advanced stages were taken into consideration (**Table 3**).

Table 3. Fischer's test

Serological reactivity for ADAM10 using 237.2 OD as cut-off value

	Pos	**Neg**	
Crc	78	28	n= 106
Cn	34	57	n= 91
CrcN0	36	16	n= 52
CrcN	42	12	n= 54

Fischer's test		**sens.**	**spec.**
Crc *vs* Cn	p<0,0001	0.74	0.63
CrcN0 *vs* Cn	p=0.0003	0.74	0.63
CrcN *vs* Cn	p<0,0001	0.78	0.64

OD= optical density; Cn= control sera; Crc= colon cancer sera; ,
N0= no tumor cells infiltrating lymphnodes;
N= tumor cells infiltrating lymphnodes
sens= sesitivity; spec= specificity

Taken together these results suggest that the serological reactivity against purified ADAM10, found in Crc patients sera can be proposed as a putative pathological marker.

3.2. Serological screening of the immunoreactivity against ADAM10 in cohorts of patients with tumours of epithelial and non-epithelial origins.

Since ADAM10 expression suggests a physiological function in the morphogenesis of several epithelial tissues, with the purpose to investigate whether the serological reactivity against ADAM10 is a feature related to the epithelial origin of the tumor tissue, cohorts from tumours of both epithelial and non-epithelial origin were tested. As tumor of epithelial origin, in addition to Crc, were tested sera from Pancreatic cancer (Pc) (n= 43) and Breast cancer (Bcr) (n=35) patients, while as malignant transformation of non-epithelial origin were tested sera from patients with hematological malignances such as B-cell chronic lymphocytic leukemia (B-CLL) (n=53) and Multiple Myeloma (MM) (n=46) (**Table 4**) (OD values for each serum are reported in *Appendix*).

Table 4. Summary of the demographic and clinical features, and the anti-ADAM10 serological reactivity of the analyzed populations.

			anti-ADAM 10 reactivity		Stage I-II	anti-ADAM 10 reactivity		Stage III-IV	anti-ADAM 10 reactivity	
	n (m/f)	Age	normalized OD	237.2 OD cut-off	n (m/f)	normalized OD	237.2 OD cut-off	n (m/f)	normalized OD	237.2 OD cut-off
Crc	106 (58/48)	67.1 sd 11.6	595.8 se 56.9	78+ 28-	52 (27/25)	560.6 se 77.47	36+ 16-	54 (31/23)	629.7 se 83.57	42+ 12-
Cn	91 (37/54)	63.8 sd 14.41	261.7 se 22.26	34+ 57-	-	-	-	-	-	-
Pc	43 (20/19)	66.77 sd 9.75	908 se 256.6	31+ 12-	-	-	-	-	-	-
Brc	35 (0/35)	51.51 sd 11.09	424.6 se 45.58	24+ 11-	-	-	-	-	-	-
B-CLL	53 (34/19)	65.55 sd 11.15	318.7 se 35.11	28+ 25-	-	-	-	-	-	-
MM	46 (21/25)	66.33 sd 10.48	298.4 se 49.21	22+ 24-	-	-	-	-	-	-

All sera were screened and normalized as for Crc sera. The frequencies of sera considered positive for the presence of antibodies against ADAM10 was significantly high both in Pc and in Brc compared with control sera (p=0.0006 and p=0.0027, respectively; Mann-Whiteney test) (**Figure 14**). On the contrary, there were no differences between sera from both patients of B-CLL and Multiple Myeloma *vs*. control subjects suggesting that the reactivity against ADAM10 was specific for tumors of epithelial origin.

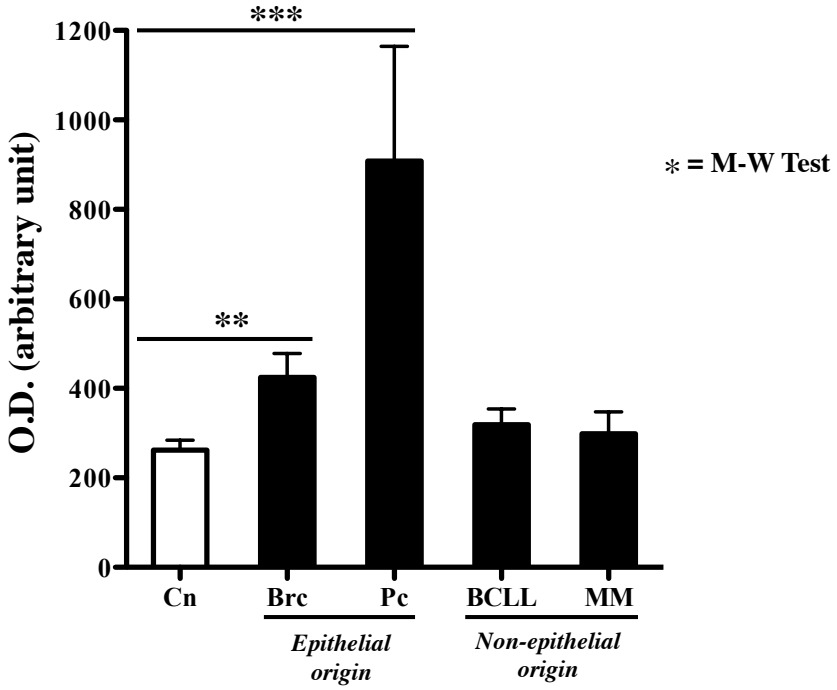


Figure 14. Analysis of the serological reactivity against ADAM10 measured as Western blot signal optical density (OD). Statistical analysis performed by Mann-Whitney (M-W) on sera from patients affected by epithelial tumors *vs*. Cn sera showed a significant presence of auto-Abs against ADAM10, both Bc *vs*. Cn and Pc *vs*. Cn. On the contrary, no differences were found on immunoreactivity against ADAM10 in patients affected by non-epithelial tumors vs. Control, either BCLL vs. Cn or MM vs. Cn (*** = p<0,0001; *** = p<0,001; ** = p<0,01; * = p<0,05) (Cn=control subjects; Bcr=Breast cancer; Pc=Pancreatic cancer; BCLL= B-cell chronic lymphocytic leukemia; MM=Multiple Myeloma).

3.3. Follow-up analysis for disease free-condition in Crc patients.

In order to see whether the presence of auto-Abs against ADAM10 was a favourable or detrimental condition for the disease progression, we analyzed for disease-free condition (no tumor relapse and no metastasis) the follow-up of Crc patients from the time of surgical resection up to 73 months. We obtained information from n=96 Crc patients that were considered as two separated groups depending on the stage of the disease (early: stage I-II; late: stage III-IV) because in patients at early stage the possibility to record an event (tumor

relapse or metastasis or patient death) is very rare. For Kaplan-Meier survival curve and Log-rank test analysis we compared the patients showing immunoreactivity against ADAM10 *vs*. the patients with no reactivity, according to the serological analysis.

The follow-up analysis of Crc patients with disease at late stages (n=49) indicated that the presence of anti-ADAM10 auto-Abs in patients prolongs significantly the disease-free condition (p=0.0034, Log-rank, test), about 14 months *vs*. 1 month at 50% of the population (**Figure 15A**). On the contrary, the follow-up analysis taking to account only the Crc patients at stages I-II (n=47) did not show enough events to be able to differentiate between Crc patients with positive and negative immunoreactivity against ADAM10 (**Figure 15A**).

Therefore the presence of auto-Abs anti-ADAM10 may be considered a favourable prognostic marker in Crc patients with infiltrated lymph nodes (stages III-IV).

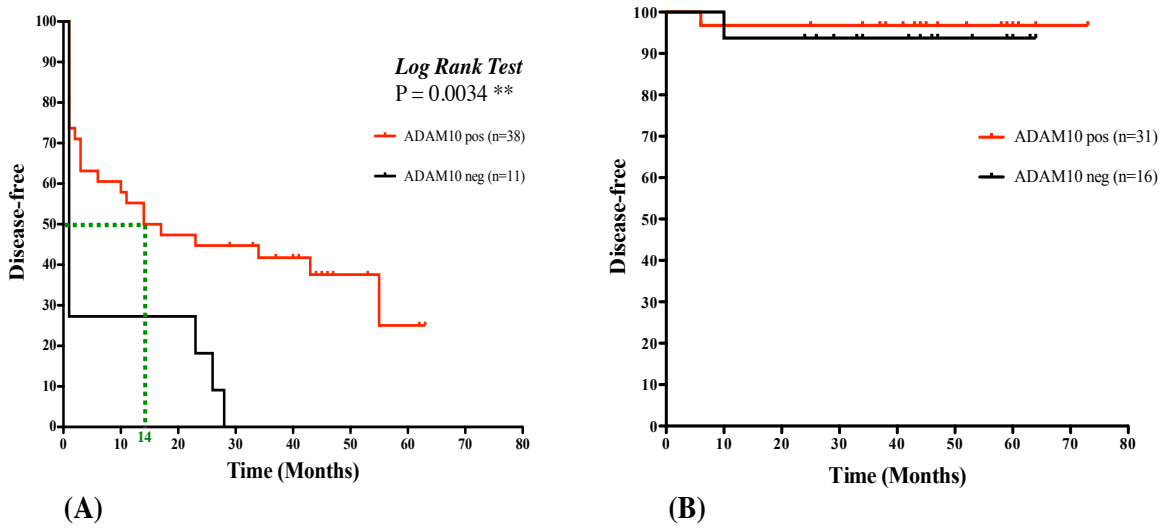


Figure 15. Kaplan-Meier analysis of disease-free condition after surgery resection in patients at stage III-IV **(A)** and patients at stage I-II **(B)**, according with the presence (ADAM10 pos) or not (ADAM10 neg) of immunoreactivity against ADAM10. The considered outcome was disease free, and the marked "event" was tumor relapse, metastasis and/or patient's death.

3.4. Evaluation of the HER2 extracellular domain released in Crc patient sera.

The metalloprotease ADAM10 is the main responsible for the extracellular domain (ECD)-shedding of the HER2 receptor, and the released ECD from the cell surface can be detected in the serum. We quantified by ELISA the HER2 ECD concentration in both Crc and Cn sera. As expected, regardless the presence of anti-ADAM10 antibodies, Crc patients showed a significant ($p<0.0001$, Mann-Whitney test) higher amount of soluble HER2 ECD in the sera if compared to control subjects (**Figure 16A**). This is consistent with an increase of cell division in a tumoral behavior. In addition, a significant ($p=0.0023$, Spearman r correlation) reverse correlation ($r= - 0.3$) between HER2 ECD released in the sera and the OD reactivity for auto-Abs against ADAM10 was observed (**Figure 16B**). Crc patients with a positive presence of anti-ADAM10 auto-Abs showed lower HER2 ECD concentrations in serum that might suggest an ADAM10-mediated inhibition of HER2 ECD shedding.

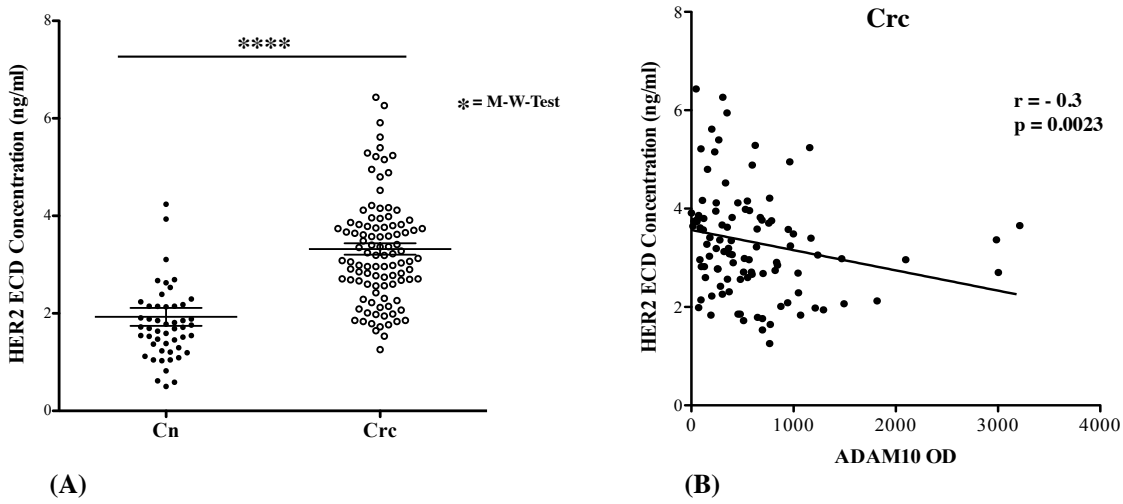


Figure 16. Evaluation by ELISA of HER2 ECD occurring in patient sera. **(A)** High HER2 ECD concentration was detected in the sera from Crc patients compare to Cn. **(B)** A reverse correlation between HER2 ECD concentration and the presence of auto-Abs against ADAM10 observed in Crc sera.

3.5. *In vitro* HER2 ECD release is inhibited by commercial anti-ADAM10 antibodies.

To confirm a possible role of auto-Abs anti-ADAM10 in inhibiting sheddase activity, the release of HER2 ECD, due to ADAM10 activity, was evaluated in LoVo metastatic colon carcinoma cell line. As shown in **Figure 17B**, LoVo cells express both pro-form and mature form of ADAM10 (98 kDa and 64 kDa respectively) as well as the HER2 in a full-length (p195) and truncated form (p95). LoVo cells also present a constitutively activated ERK1/2 signaling as reported the expression of phosphorylated ERK1/2. LoVo protein expression indicated that a large fraction of ADAM10 is constitutively activated being present as mature form, and that HER2 is also constitutively cleaved since the truncated form is present and the ERK1/2 signaling is activated as read-out of HER2 activation (**Figure 17A**). Therefore, the constitutively activated cleavage of HER2 ECD and the presence of active ADAM10 make this cellular model susceptible to inhibition by anti-ADAM10 antibody treatment. Results indicated that the HER2 ECD shedding mediated by ADAM10 was inhibited by the presence of anti-ADAM10 antibodies. Cells tested with increasing concentrations of two commercial goat anti-ADAM10 antibodies separately, resulted in a dose-dependent decrease of soluble HER2 ECD concentration detectable in culture medium (**Figure 17B)**. Control treatment with goat Immunoglobulin G (IgG) did not affect the HER2 ECD release.

Nevertheless, the Abs-treatment able to inhibit HER2 ECD release did not significantly affected LoVo cell proliferation nor seemed to affect the intracellular signaling pathway as assessed by ERK1/2 phosphorylation (data not shown).

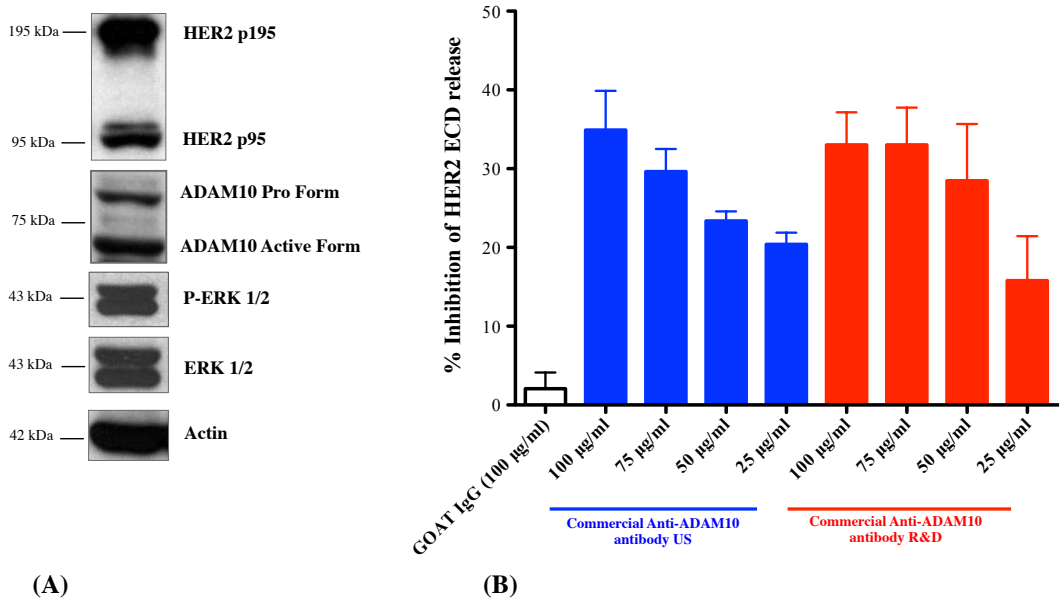


Figure 17. (A) Protein expression in the metastatic Crc LoVo cell line. **(B)** LoVo cell line was treated for 72 hours with increasing concentrations (25 µg/ml, 50 µg/ml, 75 µg/ml and 100 µg/ml) of two commercial goat anti-ADAM10 antibodies (US Biological and R&D systems) or IgG purified from goat serum (100 µg/ml) as control. Culture supernatants were collected and assayed by ELISA for the presence of soluble HER2 ECD.

3.6. Commercial Anti-ADAM10 antibody blocked the migration of LoVo cell line.

Epithelial cell migration was analyzed in absence or presence of commercial anti-ADAM10 antibody by wound healing migration test. The LoVo Crc cell line expressing ADAM10 was incubated with commercial goat anti-ADAM10 Ab (10 µg/ml). After 72 hours LoVo cells showed a marked inhibition of the wound closure if compare with the cells incubated with IgG purified from goat (10 µg/ml) tested as control (T-test $p<0.0045$) (**Figure 18**). These results suggested that the possible interference of antibodies anti ADAM10 with ADAM10 functions results in a reduction of migratory properties of the tumoral cells.

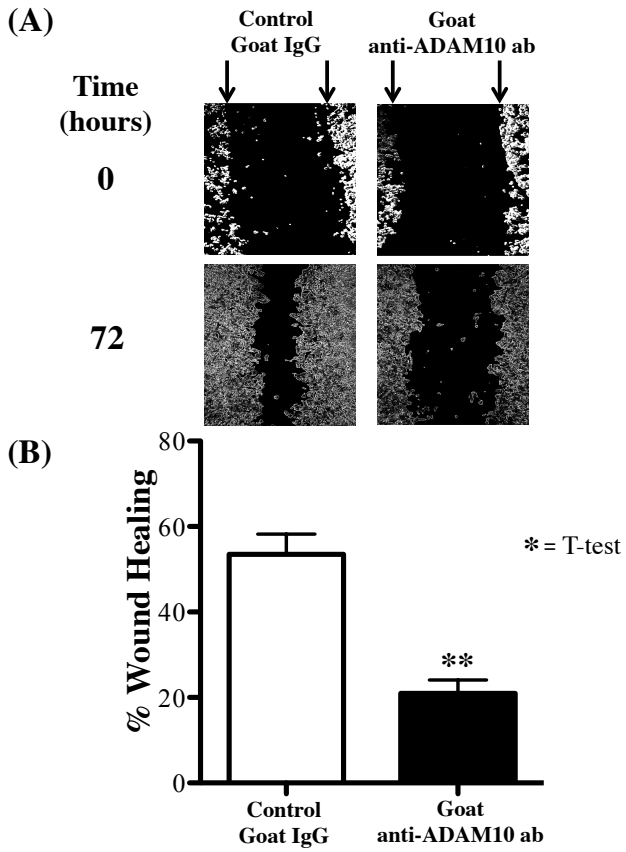


Figure 18. Wound healing cell migration assay. **(A)** A representative experiment showed the inhibitory effect of the commercial goat anti-ADAM10 Ab (US Biological; 10 μg/ml) on LoVo cells migration. **(B)** After 72 hr. the presence of anti-ADAM10 Ab inhibited more than 50% of the wound closure.

3.7. *In vitro* effects of IgG purified from Crc patients sera on migration and HER2 ECD release.

The sera IgG fraction from representative Crc patients (6 positive and 6 negative for anti-ADAM10 serological reactivity) were purified on G-agarose and, subsequently, tested for purity in SDS-PAGE. The ponceau staining showed the presence of both light (25 kDa) and heavy (50 kDa) chains of the IgGs (**Figure 19**).

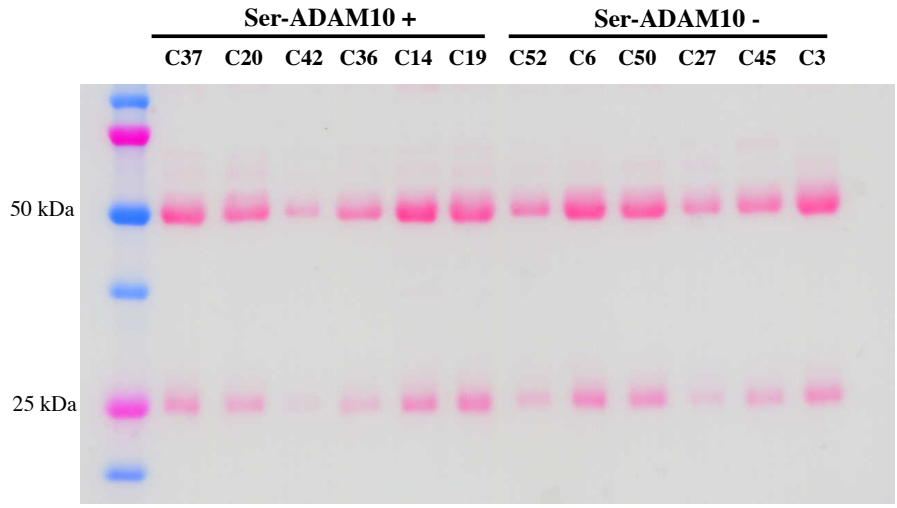


Figure 19. IgG purified from Crc patients sera. Five µl of purified IgG from Crc patients with positive (Ser-ADAM10 +) (C37, C20, C42, C36, C14, C19) and negative (Ser-ADAM10 -) (C52, C6, C50, C27, C45, C3) anti-ADAM10 serological reactivity were resolved by SDS-PAGE. Ponceau red staining showed both heavy (50 kDa) and light (25 kDa) chains of the IgGs.

In order to be sure that the purified IgGs retained the specific reactivity, they were tested for reactivity against purified rhADAM10, the results confirmed that the purified IgG preserved the immunoreactivity against rhADAM10 tested in the Crc serological screening (**Figure 20**).

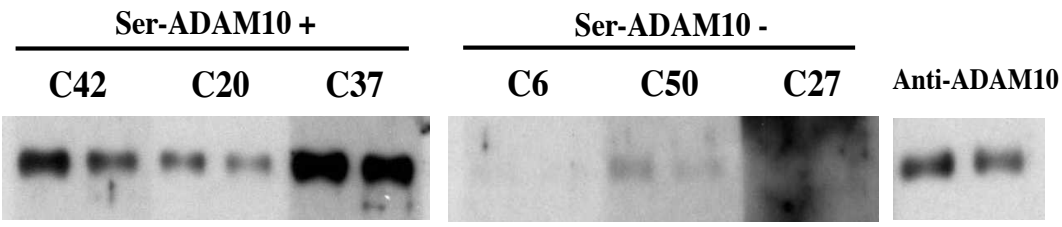


Figure 20. Representative immunoreactivity of the IgGs purified from Crc patient sera on rhADAM10 (in duplicate), showing the retention of the specific serologic reactivity as recorded before IgGs purification.

The purified IgGs from Crc patients sera were used to evaluate whether, similarly to the commercial anti-ADAM10 Abs, might affect the functions of LoVo cell line expressing ADAM10. Wound healing test and HER2 ECD release assay were carried out.

Wound healing experiment was performed to test the effect of the purified IgGs on cell migration. After 72 hours of treatment, cell incubated with IgGs (10 μg/ml) from both Ser-ADAM10 + patients and Ser-ADAM10 – patients did not show significant differences in cell migration neither between them nor if compared with the cells incubated with medium alone (**Figure 21A**).

HER2 ECD release assay was performed in order to evaluate the effects of the purified IgGs on the ADAM10-sheddase activity. LoVo cells were incubated for 72 hours with IgGs (100 μg/ml) from both Ser-ADAM10 + patients and Ser-ADAM10 – patients. Soluble HER2 ECD released in the medium was measured by ELISA, and the results showed a concentration of HER2 ECD barely lower in cells treated with IgGs from patients compared to cells incubate with medium alone, which suggested a weak inhibition of the ECD release. However, IgGs purified from patients ADAM10 + did not inhibit in a significative manner the ECD release if compare with the cells treated with IgGs from patients ADAM10 – (**Figure 21B**).

In conclusion, the IgGs from Ser-ADAM10 + patients resulted not to be effective in inhibiting both tumoral cell migration and ADAM10 sheddase activity.

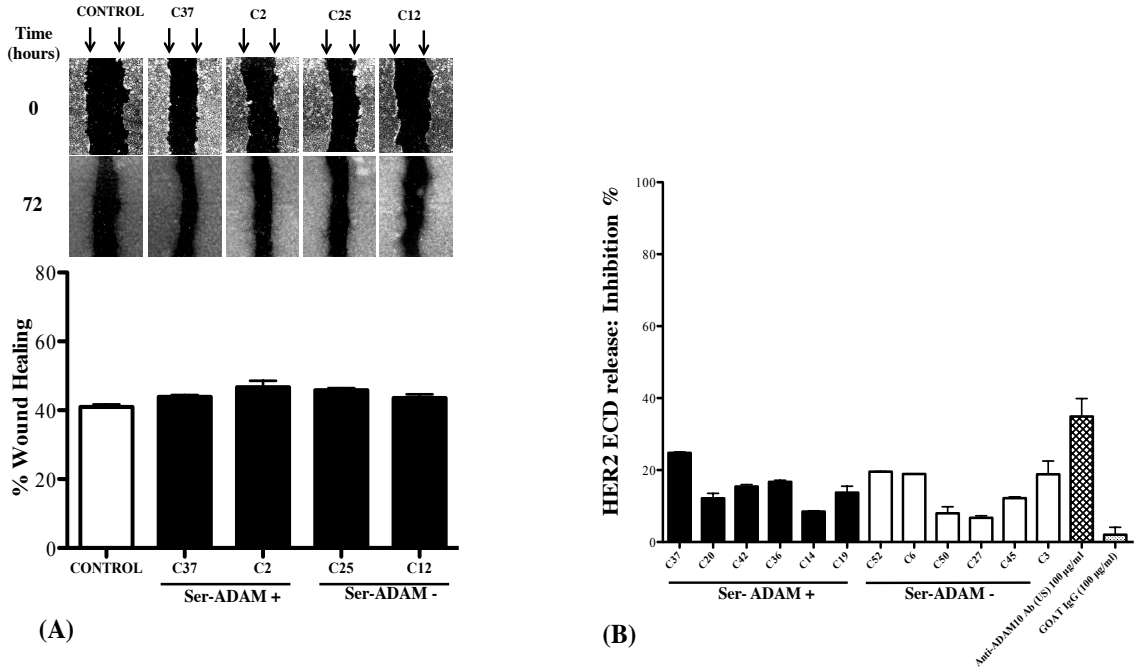


Figure 21. LoVo cell line incubated with IgGs purified from Crc patients sera with a positive (Ser-ADAM10 +) or negative (Ser-ADAM10 -) immunoreactivity against ADAM10. **(A)** Wound healing experiment showed no significant inhibition of cell migration; results were calculated as the percentage of closed wound area referred to the initial total area of the wound **(B)** Evaluation by ELISA of HER2 ECD released in the culture medium. No significant differences in HER2 ECD release were observed between cells treated with IgGs purified from Ser-ADAM10 + patients compared to cell treated with IgGs purified from Ser-ADAM10 – patients; HER2 ECD levels are expressed as percent of inhibition referred to control cells treated with medium alone.

3.8. Expression of ADAM10 and proteins targets of its sheddase activity in primary tumor tissues from Colorectal cancer patients.

With the aim of to evaluate the presence of different isoforms of ADAM10 in Crc primary tumor tissues, namely the mature active isoform of about 98 kDa and the inactive pro-protein of about 64 kDa, Western blot analysis was carried out on tumoral tissues from seven Crc patients. The samples include five patients with positive immunoreactivity against ADAM10 and two with showed no auto-Abs anti-ADAM10. The precursor (uncleaved) and

mature (cleaved) isoforms of ADAM10 were differentially expressed in Ser-ADAM10 + patients compared to Ser-ADAM10 - patients.

The tumors from Ser-ADAM10 + patients showed the expression of equal amount of mature and the immature pro-form of ADAM10, while the Ser-ADAM10 – patients showed a prevalent expression of the active mature ADAM10 (**Figure 18**). Then, we tested the expression of the HER2 receptor, the target of the ADAM10 sheddase activity, and the activation status of ERK1/2, a molecule downstream to the HER2 activation pathway, since it correlates with HER2 activation.

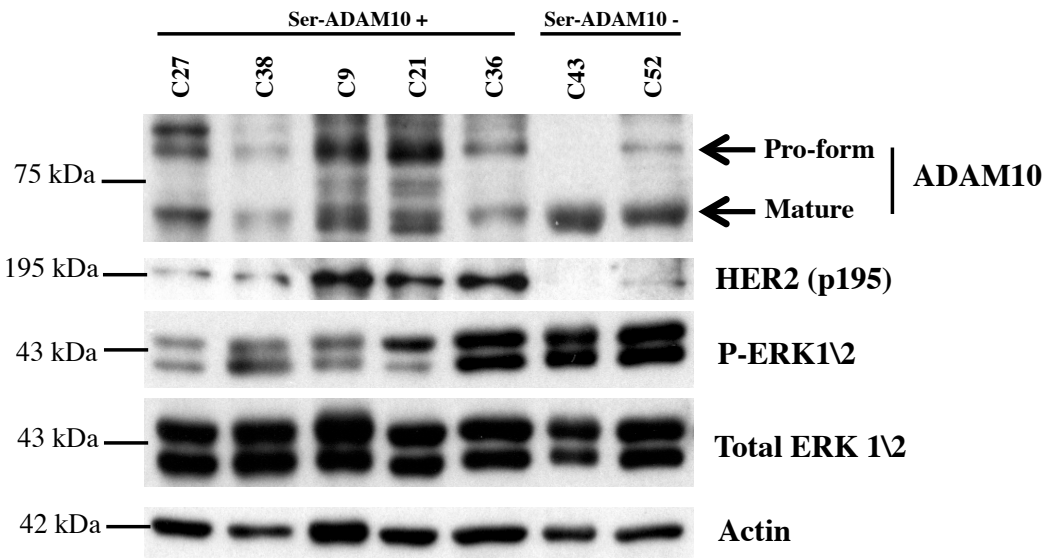


Figure 18. Western blot analysis on colon specimens from Crc patients (patients with a positive immunoreactivity against ADAM10: C27, C38, C9, C21, C36; patients without immunoreactivity against ADAM10: C43, C52) for the expression of ADAM10 and its related proteins HER2, Total ERK1/2 and phosphorylated-ERK1/2. Reactivity with anti-β-actin mAb was used as a protein-loading control.

Accordingly to the predominant expression of the active ADAM10 mature form, the Ser-ADAM10 – patients showed faint or no expression of the HER2 (p195) isoform,

possibly due to an increased HER2 ECD cleavage, that was confirmed by the activation of ERK1/2 signaling as indicated by strong ERK1/2 phosphorylation (**Figure 18**). On the contrary, Ser-ADAM10 + patients showing increased expression of immature ADAM10 (inactive protein) reported an higher expression of the inactive HER2 (p195) isoform and a decreased expression of phosphorylated-ERK1/2, suggesting a lower activity of ADAM10 on e HER2 ECD cleavage.

4. DISCUSSION

In cancer, TAAs are used in diagnosis as early detection biomarkers reporting in advance that malignancy transformation has been started in the organism and the immune system produce auto-Abs against these TAAs in an effort to counteract the tumor [8]. In the case of Crc, the presence of polyp or adenoma reports the beginning of abnormal cell growth and even if they are not able to spread beyond of the mucosa yet, they are able to elicit an immune response. In particular, in a previous work in the laboratory, with the aim to identify proteins eliciting humoral response in Crc patients, the membrane metalloprotease ADAM10 was identified as a putative TAA by a proteome-based approach (SERPA).

In the present study, ADAM10 was validated as TAA in a large number of Crc sera and control sera confirming the presence of a significant immunoreactivity anti-ADAM10 in Crc patients compared to healthy subjects. Moreover, in order to investigate whether anti-ADAM10 serological reactivity is a specific feature of Crc or is a characteristic shared with others tumors, we found that also Pc and Brc, which are tumor of epithelial origin, elicited a significant anti-ADAM10 immunoreactivity, while sera from patients affected by hematological malignancies did not. This is consistent with the fact that ADAM10 play a critical physiological function in the epithelial cell development [43]. ADAM10 regulates several morphogenetic movements during embryogenesis such as cell proliferation, differentiation and remodeling of the extracellular matrix (ECM), vascularization and cell migration [43, 72]. These events occur several times during organogenesis in both normal development and during tumor progression [72]. Since the presence of ADAM10 is indispensable in the epithelial morphogenesis, any aberrant modification of the protein might elicit the immunorreactivity against ADAM10. Therefore, the presence of auto-Abs

against ADAM10 in patients sera might be a biomarker candidate for carcinomas of epithelial origin.

In particular, the disease course of the Crc patients with advanced stages, when the lymph nodes were infiltrated by the tumor cells, showed that the presence of auto-Abs anti-ADAM10 prolonged significantly the disease-free condition. This was not occurring for Crc patients at early stages suggesting that the infiltrated lymph nodes is a critical point of the disease where the humoral response might confer an advantage in limiting the progression of the tumor. In fact, ADAM10 has been reported to be overexpressed in the advanced stages of the Crc [22], therefore it seems that is in these stages that the auto-Abs may have a crucial role in affecting tumor growth.

Since a favourable condition of the Crc patients with positive immunoreactivity against ADAM10 was observed, we hypothesized that the auto-Abs might recognized some specific part of the protein and inhibit its activity. Thereby, we focused on the possible effects that the auto-Abs anti-ADAM10 might have in Crc patients and on the possible correlation between the presence of high immunoreactivity and progression of the disease. The major activity of ADAM10 is known to be located at the metalloprotease domain that promotes the shedding of several membrane bound receptors [27], in particular ADAM10 is the major metalloprotease responsible for the ECD-cleavage of the HER2 receptor [37], which in turn became activated leading to cell proliferation [66]. The presence of HER2 ECD released in serum is used as marker for Breast cancer metastases [73, 74] that indicate a poor prognosis. Therefore the observation that Ser-ADAM10 + patients showed lower concentrations of HER2 ECD in serum suggests that the auto-Abs anti-ADAM10 might inhibit *in vivo* the sheddase activity of ADAM10 in Crc patients. This hypothesis was confirmed *in vitro* when LoVo Crc cell line treated with two commercial anti-ADAM10 antibodies showed a dose-dependent reduction of the amount of HER2 ECD released in the

culture medium. Therefore, consistently with the physiological function of the HER2 receptor, the inhibition of the HER2 cleavage should promote also an inhibition of cell division [66], but we did not observe any decrease of either cell proliferation or phosphorylation of ERK1/2 (as read-out of reduced cell activation [66]). A possible explanation is that LoVo is a cell line with a constitutively activated ERK1/2 signaling (**Figure 17A**), thus the inhibition of the HER2 activation was not able to inhibit also, at least *in vitro*, the activation signaling which was already started.

ADAM10 is also able to influence cell adhesion and cell-cell interactions inducing the remodeling of the extracellular matrix (ECM) [61]. *In vitro* migration experiment using LoVo Crc cell line treated with commercial anti-ADAM10 antibody resulted to be effective in inhibiting cell migration. This might be of relevance since cell migration drives tumor metastasis during malignant transformation [60] and its inhibition might slow down the progression of the disease explaining the favorable prognosis of Ser-ADAM10 + patients.

These experiments indicated that the inhibition of ADAM10 results in a decrease of its sheddase activity *in vitro*. Therefore, *in vivo*, the decreased sheddase activity that occurs in patients might be due to a blocking effect on ADAM10 by the auto-Abs. In alternative, a possible explanation is that the detected immunoreactivity is a read-out signal elicited by aberrant modifications of the ADAM10 protein, and that such modifications make ADAM10 non-functional in the absence of any role for the auto-Abs. Thus, in order to evaluate *in vitro* the effects of the auto-Abs against ADAM10, IgG fractions were purified from sera of representative Crc patients and were used both on HER2 ECD-shedding assay and on wound healing experiment. The aim was to differentiate between the effects of purified IgGs from Ser-ADAM10 + and Ser-ADAM10 – patients. We were expecting a significant high inhibition by purified IgGs from Ser-ADAM10 +, however, despite the successful IgG purification from the sera and the fact that they retained the specific

immunoreactivity against ADAM10, these Abs resulted not to be effective in inhibiting LoVo cell migration. In the case of HER2 ECD release assay, the purified IgG showed a pattern of inhibition which, however, did not correlate with Ser-ADAM10 + patients and probably was due to a non-specific effect of the Abs. These results might be explained owing to the low amount of Ig fraction specific for ADAM10 present in the total amount of purified IgG. An alternative explanation is that the auto-Abs anti-ADAM10 were not effective because LoVo cell line did not express the specific epitope that elicited the serological reactivity in Crc patients. The recognition of tumor-specific epitopes presented in the anomalous protein may be due to changes in the protein structure, in post-translational modifications and in the expression of different isoforms heterogeneously occurring during tumoral transformation [11, 19, 67]. In fact, Western blot analysis on cell lysates obtained from patients specimens showed different expression of ADAM10 between Ser-ADAM10 + and Ser-ADAM10 – patients. The expression of immature ADAM10 isoform (98 kDa) is predominantly expressed in Ser-ADAM10 + patients while its expression in Ser-ADAM10 – patients is low or absent. The overexpression of immature (non cleaved) inactive ADAM10 in Crc patients might be due to its reduced processing in the Golgi compartment, resulting in a reduced expression of active ADAM10 in the surface membrane. Accordingly, the ADAM10 sheddase activity is reduced on the membrane of these cells, explaining the reduced HER2 ECD release and decreased expression of phosphorilated-ERK1/2 detected in these patients. Thus, the serological reactivity elicited by ADAM10 in Crc patients might report the overexpression of inactive ADAM10, in particular we might hypothesize that the specific sequence recognized by the auto-Abs might be an epitope within the non-cleaved prodomain. This might explain why purified IgG from Crc patients were not able to confirm *in vitro* the successful inhibition of the ADAM10 activity observed using commercial anti-ADAM10 antibodies. The purified IgG were not able to recognize the specific epitope since

LoVo cells, as well as any living cell, do not express on the cell surface the inactive ADAM10 containing the prodomain, being the enzymatic precursor processed in intracellular compartment [76]. The intracellular localization in which the recognition and cleavage of ADAMs substrates occurs is still a matter of debate [75], however the surface form of ADAM10 appears to be processed while the majority of the proenzyme form of ADAM10 is found in the Golgi [76]. Nowadays it is not completely clear how antibody response against intracellular proteins occurs. Some mechanisms that allow intracellular peptides to trigger the immune response have already been described, such as chaperon-mediated autophagy [68] and exosomes [69]. Moreover the aberrant tumor cell death is an accepted hypothesis in which the intracellular aberrant proteins are released from tumor cells [70, 71]. The knowledge of the modificated part of the TAA that is recognized by the auto-Abs might be useful as therapeutic target but also to understand the possible secondary mechanism that is involved in the protein processes.

In conclusion, the presence of auto-Abs anti-ADAM10 may be a favourable prognosis biomarker for Crc patients at advanced stages of the disease that report an overexpression of inactive ADAM10 during the cell transformation and accordingly the reduce protein maturation lead to a decrease of its sheddase activity that decline the proliferative and invasive capacity of tumor cells. Patients belonging to the same stage of the disease may evolve in different manner leading to different prognosis, depending on the autoantibody response that has been developed. Therefore, from the clinical point of view, the relevance of this auto-Abs provides information about the disease progression which is crucial in order to perform personal therapies, based on the immune response elicited against ADAM10, with the aim to suppress tumor or slow down its progression.

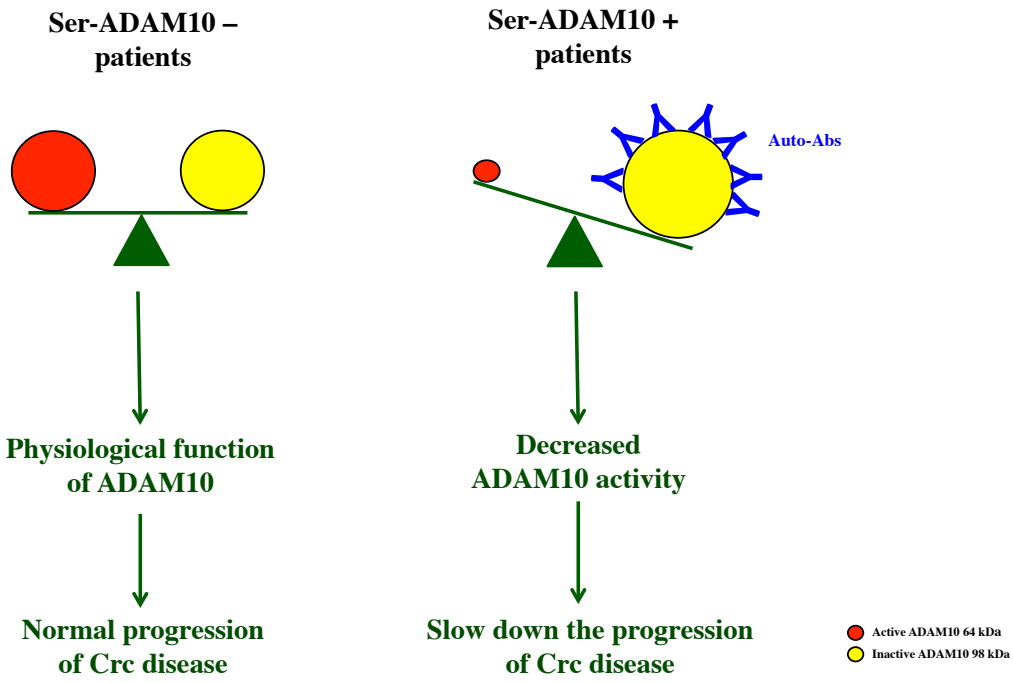


Figure 19. Schematic representation of our hypothesis. Ser-ADAM10 – patients present a normal expression of both active ADAM10 and inactive ADAM10 resulting in a physiological function of the protein which promote the progression of the disease in Crc patients. On the contrary, Ser-ADAM10 + patients show an overexpression of the inactive ADAM10 that elicit the immune response and is recognized by the auto-Abs, then the reduced expression of the active form of ADAM10 is responsible for the decrease in the activity of the protein and results in slowing of the disease progression.

5. MATERIALS & METHODS

5.1. Sera and Tissue Samples

Samples were obtained following informed patient consent and after approval by the Institutional Ethical Committee. Sera were collected at the time of surgical resection from a cohort of 106 patients with Colorectal cancer (Crc), and from a control group (Cn) that included 91 healthy subjects and patients with non-tumoral pathologies. Crc patients with (Stage III and IV; n= 54) or without (Stage I and II; n= 52) infiltration in the regional lymph nodes were considered as separated subgroups in some analyses. Cohorts of sera from different tumors namely pancreatic carcinoma (Pc, n= 43), breast carcinoma (Brc, n= 35), B-cell chronic lymphocytic leukemia (B-CLL, n= 53) and multiple myeloma (MM, n= 46) were also collected. The summary of the demographic and clinical features of the populations enrolled in the study and features of the single subjects of each group are reported in the *Appendix*. Tissue specimens from Crc tumor were frozen immediately after surgical removal and stored at -80^{0}C until use.

5.2. Cell line and cell culture

The tumor cell line used in this study was LoVo metastatic Colon carcinoma (American Type Culture Collection, ATCC); the cells were grown in RPMI containing 10% fetal bovine serum (FBS), penicillin, and streptomycin, under standard conditions (37^{0}C, 5% CO_2) in humidified incubator.

5.3. Serological screening on purified ADAM10: SDS-PAGE and Western blot analysis.

Recombinant purified extracellular domain of mouse recombinant ADAM10 (Calbiochem), sharing 97% amino acid homology with human ADAM10**,** was resolved (200 ng lane) by 10% acrylamide SDS-PAGE (Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis). Proteins were electrotransferred to nitrocellulose membrane that was cut vertically into strips that contained two lines of protein and blocked by incubation with fat-free milk for 1 hour at room temperature. After that, each strip was incubated at 4°C over night with sera obtained from either patients or controls (1:5000 dilution). As secondary antibodies were used either goat anti-human IgG (Southern Biotechnology Associates, Birmingham, AL) or rabbit anti-goat (Dako Denmark) conjugated with horseradish peroxidase. The signals were developed by chemiluminescence substrate reaction by using ECL (GE-Healthcare) followed by autoradiography with film exposure. In order to quantify the sera reactivities, films were used for densitometric analysis. Images were acquired using a Personal SI Laser Densitometer (Molecular Dynamics), and the analysis of the optical density (OD) of sera reactivity was performed using Image Quant v.1 software (GE-Healthcare). The signals were normalize with a positive control obtained by using a commercial anti-ADAM10 antibody (Goat anti-human ADAM10 Ectodomain Antibody. R&D Systems) tested in all experiments.

5.4. HER2 ECD release assay by ELISA.

The quantification of HER2 ECD (p105) in both cell culture conditional medium and serum from patients and healthy subjects was measured by using cerb-B2/c-neu rapid format ELISA kit (Calbiochem).

The ELISA kit was used to measured the HER2 ECD released in the conditioned medium of LoVo carcinoma cell line culture. Cells were seeded in 96-well plates at $5x10^3$ cells/well in 100 µl of RPMI supplemented with 10% FBS. Preliminary test experiment confirmed that the ELISA kit was not reactive against the possible presence of HER2 from the FBS. After 24 hours, the medium was replaced with or without the addition of antibodies (goat anti-ADAM10 antibody US Biological, goat anti-ADAM10 antibody R&D Systems, purified IgG from goat serum or purified IgG from Crc patient sera) at the concentrations indicated in individual experiments. Cells were cultivated for further 72 hours. Cell culture conditioned medium were removed, centrifuged to discard any cells and concentrated with centrifuge 50 kDa cut-off device filters (Amicon) before use in ELISA test.

Recombinant full length p195HER2/neu (p195) was used to generate the standard curve (0 ng/ml, 0.188 ng/ml, 0.375 ng/ml, 0.75 ng/ml and 1.5 ng/ml). The reaction volume for each sample and each standard was 100 µl/well. The ELISA assay was performed on 96 well plates coated with mouse monoclonal HER2 antibody that recognizes the extracellular domain. The detector antibody, was a biotinylated mouse monoclonal anti-HER2 and the reactivity was revealed by streptavidin-peroxidase followed by the Substrate tetra-methylbenzidine (TMB) addition. The color intensity, proportional to the HER2 ECD concentrations, was measured with a spectrophotometer adjusted at dual wavelengths of 450/595 nm, within 30 min from adding the Stop Solution. All reagents are included in the ELISA kit.

The ELISA kit was also used to measure the HER2 ECD released in the sera from Crc patients and control subjects.

All samples and standards were diluted (1:20) using Sample diluent and were assayed in triplicate.

5.5. Protein expression in Primary Tumor Tissues.

Tissue species from -80^{0}C storage were immerse in liquid nitrogen and mechanically broken in a Eppendorf tube by using a dedicated pestle. Then extraction buffer (50mM Tris HCl pH 7.4, 150mM NaCl, 1%Triton, 1mM EDTA) containing protease (1:100 protease inhibitors cocktail SIGMA) and phosphatase inhibitor (1mM NaF, 2mM Na_3VO_4) was added and samples were incubated in ice for 20 min. After centrifugation (16000g, 20 min, 4^{0}C) the supernatant was recovered and solubilized protein concentration was determined by Bradford method (Bio-Rad Protein Assay) using bovine serum albumin (BSA) as standard. Total proteins from each sample (15μg) were loaded on 10% acrylamide gel SDS-PAGE. Once the electrophoresis is finished, proteins were electro transferred to nitrocellulose membrane and blocked by incubation with BSA for 1 hour at room temperature. Then, the membranes were incubated over night at 4^{0}C with the following primary antibodies: mouse anti-ADAM10 monoclonal antibody (Abcam), rabbit anti-HER2 polyclonal antibody (Abcam), rabbit anti-phospho-p44/42 MAPK (ERK1/2) antibody (Cell signaling), rabbit anti-total p44/42 MAPK (ERK1/2) antibody (Cell signaling), mouse anti-β-actin monoclonal antibody (Sigma). As secondary antibodies were used goat anti-rabbit Ig and rabbit anti-mouse Ig conjugated with horseradish peroxidase (Dako Denmark). The protein loading and transfer was normalized by Ponceau S (Sigma) staining and by actin immunoblotting.

5.6. Immunoglobulin G purification from Crc patient sera and goat serum.

Total IgG were purified from sera of colon cancer patients, either positive or negative for the presence of anti-ADAM10 Abs, and from goat serum, the latter as to be used as negative control of commercial goat anti-ADAM10 antibodies. IgG purification was performed by using Recombinant Protein G Agarose (rProtein G Agarose; Invitrogen).

According to the IgG binding capacity of rProtein G Agarose (20mg/ml) and the IgG concentration range in human (7.5-22 mg/ml in human and 18-24 mg/ml in goat), 200 µl of dry beads were used to purified IgG from 250 µl of serum. After washing with phosphate buffered saline (PBS) (3x) the dry beads were mixed with 250µl of serum and the volume was adjusted to 1 ml with PBS. The mixture was left to incubate for 24 hours at 4°C under gently shacking. The supernatant was then removed, the beads were washed 3 times with 1 ml of PBS, the bound IgGs were eluted by incubation with 1ml of Elution Buffer (0.1 M Glycine HCL, pH 2.6) at 20^0C for 10 min. The pH was then adjusted to about pH 7.0 by the addition of 16.7 µl (1:60 v/v) of Tris 1M, the beads were pelleted by centrifugation (2 minutes, 13000 rcf, 4°C) and the eluted IgGs were concentrated using centrifugal 100kDa cut-off device filters (Amicon) (20 min, 13000 rcf, 4°C). By using the same device, the buffer in which the purified IgG were resuspended, was exchanged and concentrated to reach a final volume of 35 µl in PBS for each samples. Total IgG concentration was determined by Bradford method (Bio-Rad Protein Assay) using BSA as standard.

5.7. *In vitro* wound healing assay.

LoVo cells were seeded in 12–well plates at $5x10^5$ cells/well in 1 ml of RPMI containing 10% FBS and cultured overnight as to reach cell confluence. The next day, a straight scratch was done by using a pipette tip at right angle with the plate in order to maintain the width wound uniform. Cells were washed with PBS and subsequently 500 ul of medium containing either 10 µg/ml of commercial goat anti-ADAM10 antibody US Biological or 10 µg/ml of IgG purified from Crc patients. Medium with 10 µg/ml of purified IgG from goat serum was tested as control. The media were replaced by fresh medium containing the same treatment concentrations every 24 hours. To monitor cell migration, images (10x objective) of the wound closure were taken at times 0 and 72 hours (Leica

DFC300 FX, Wetzlar, Germany) using a 10x objective. The streaks of each plate were photographed on three different areas and the wound area was measured with the informatics software ImageJ (Imaje Processing And Analysis in Java). The closing percentage after 72 hours was calculated with respect to Time 0 for each condition.

5.8. Statistical analysis

Each serum was tested twice on purified ADAM10 and the reactivity was quantified by comparing normalized OD in patients and controls. After background subtraction evaluated for every sera, OD values were normalized to the reactivity of the commercial anti-ADAM10 obtained in the same film exposures. Statistical analysis of categorical data (positive or negative sera immunoreactivity) was performed by 2x2 contingency table analysis using Fisher's exact test and two-tailed p-value. Wound healing measures were evaluated using unpaired Student's t-test with two-tailed p value for the comparison of two means with standard deviation. In the case of non-Gaussian distribution (intensity of immunoreactivity as arbitrary unit of OD and HER2 ECD release concentration in sera), data were evaluated using Mann-Whitney U test to compare differences between two independent groups and the analysis of variance was performed using the Kruskal-Wallis test. The follow-up of Crc patients was evaluated by generating Kaplan-Meier survival curves using tumor-relapse, presence of metastasis and patient death as end-point; survival curves were compared using the log-rank test. The receiver operating characteristic curve (ROC) was used to define the threshold value for OD that gave the better ratio between sensitivity and specificity. Correlation analysis was evaluated using Spearman's rank coefficient. Statistical analysis were performed using GraphPad Prism V5 software (GraphPad Inc.), in all analysis $p<0.05$ was considered to be statistically significant.

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7. APPENDIX

Supplemental Materials Table S1

Demographic and clinical features of the Colorectal cancer patients.

Ptz #	Gender	Age	Stage (category)[b]	anti-ADAM 10 reactivity		Follow-up
				normalized OD	237.2 OD cut-off	
1	m	53	Stg I (T1N0M0)	228,52	-	A 24
2	f	57	Stg I (T1N0M0)	1157,94	+	A 52
3	f	72	Stg I (T1N0M0)	819,881018	+	A47
4	m	41	Stg I (T1N0M0)	85,368708	-	A46
5	m	71	Stg I (T1N0M0)	399,889648	+	A6
6	f	60	Stg I (T1N0M0)	1493,091095	+	A43
7	m	65	Stg I (T1N0M0)	625,581678	+	A41
8	m	78	Stg I (T2N0M0)	152,418569	-	A47
9	f	54	Stg I (T2N0M0)	1044,74624	+	-
10	m	77	Stg I (T2N0M0)	1,048112	-	A26
11	f	85	Stg I (T2N0M0)	1211,657354	+	-
12	f	73	Stg I (T2N0M0)	408,694948	+	A45
13	f	70	Stg I (T2N0M0)	137,490284	-	A44
14	f	62	Stg I (T2NxM0)	1293,028738	+	A43
15	m	85	Stg I (T2N0M0)	284,229006	+	A37
16	m	50	Stg I (T2N0M0)	243,319154	+	A42
17	m	57	Stg I (T2N0M0)	159,6	-	A 60
18	m	83	Stg I (T2N0M0)	242,94	+	A 33
19	m	73	Stg I (T2N0M0)	84,55	-	A 34
20	f	61	Stg I (T2N0M0)	46,34	-	A 53
21	m	71	Stg I (T2N0M0)	122,09	-	A 29
22	m	66	Stg I (T2N0M0)	390,35	+	-
23	f	73	Stg II (T3N0M0)	364,93	+	A 34
24	m	73	Stg II (T3N0M0)	785,73	+	M 6 +32
25	f	60	Stg II (T3N0M0)	117,89	-	A 63
26	f	53	Stg II (T3N0M0)	71,82	-	A 64
27	m	84	Stg II (T3N0M0)	14,83	-	A 60
28	m	80	Stg II (T3N0M0)	182,24	-	A 59
29	f	57	Stg II (T3N0M0)	372,12	+	A 59
30	m	81	Stg II (T3N0M0)	529,93	+	A 58
31	m	61	Stg II (T3N0M0)	565,57	+	A 61
32	f	51	Stg II (T3N0M0)	455,41	+	A 73
33	f	63	Stg II (T3N0M0)	569,43	+	A 60
34	f	62	Stg II (T3N0M0)	596,5	+	A 59
35	f	75	Stg II (T3N0M0)	707,75	+	A 64
36	m	59	Stg II (T3N0M0)	127,313061	-	-
37	f	77	Stg II (T3N0M0)	675,954563	+	A47
38	f	72	Stg II (T3N0M0)	70,683728	-	-
39	m	84	Stg II (T3N0M0)	478,155141	+	A45
40	m	64	Stg II (T3N0M0)	1068,601845	+	A25
41	f	79	Stg II (T3N0M0)	458,15559	+	A45
42	f	74	Stg II (T3N0M0)	398,354127	+	A45
43	m	80	Stg II (T3N0M0)	1047,594678	+	A44
44	m	78	Stg II (T3N0M0)	551,133175	+	A44
45	f	78	Stg II (T3N0M0)	3003,212479	+	A43
46	f	96	Stg II (T3N0M0)	773,399173	+	A43
47	f	52	Stg II (T3N0M0)	279,522874	+	A43
48	m	72	Stg II (T3N0M0)	267,584664	+	A41
49	m	74	Stg II (T3N0M0)	2098,17	+	A 38
50	f	70	Stg II (T4N0M0)	25,42	-	M 10 + 28

51	m	82	Stg II (T4N0M0)	1169,88	+	A 64
52	m	59	Stg II (T4N0M0)	691,37	+	A 59
53	m	68	Stg III (T2N1M0)	301,18	+	-
54	f	55	Stg III (T3N1M0)	199,54	-	M 0 +23
55	f	61	Stg III (T3N1M0)	178,14	-	R 28 M 28 A38
56	f	68	Stg III (T3N1M0)	518,75	+	A 63
57	f	69	Stg III (T3N1M0)	548,63	+	M 17
58	m	79	Stg III (T3N1M0)	674,81	+	M 34
59	m	80	Stg III (T3N1M0)	998,23	+	R 11 M 35
60	m	73	Stg III (T3N1M0)	702,51	+	M 55 A55
61	m	67	Stg III (T3N1M0)	843,53	+	M 3 +21
62	m	66	Stg III (T3N1M0)	1237,61	+	A 33
63	f	74	Stg III (T3N1M0)	949,55	+	A 62
64	m	90	Stg III (T3N1M0)	1470,67	+	M 10 + 30
65	m	47	Stg III (T3N1M0)	648,035927	+	R43 A47
66	m	70	Stg III (T3N1M0)	1818,219038	+	A45
67	m	49	Stg III (T3N1M0)	695,339305	+	A45
68	m	70	Stg III (T3N1M0)	199,52876	-	R16 A45
69	m	73	Stg III (T3N1M0)	352,800146	+	A44
70	f	80	Stg III (T3N1M0)	767,002277	+	R23 +23
71	f	65	Stg III (T3N1M0)	308,258811	+	A41
72	f	48	Stg III (T4N1M0)	3,91	-	M 23 A31
73	f	52	Stg III (T4N1M0)	369,47	+	-
74	f	83	Stg III (T2N2M0)	54,65	-	M 26 +30
75	m	58	Stg III (T2N2M0)	766,55	+	-
76	m	81	Stg III (T3N2M0)	59,94	-	M 0 +14
77	m	44	Stg III (T3N2M0)	510,18	+	-
78	f	73	Stg III (T3N2M0)	644,22	+	M 2 +12
79	m	64	Stg III (T3N2M0)	334,4	+	A 37
80	m	69	Stg III (T3N2M0)	261,01	+	A 53
81	f	44	Stg III (T3N2M0)	587,98	+	M 3 A 35
82	m	66	Stg III (T3N2M0)	834,34	+	M 3 +27
83	m	76	Stg III (T4N2M0)	351,84	+	M 6 +10
84	m	45	Stg III (T4N2M0)	757,57	+	M 0 +28
85	f	77	Stg III (T4N2M0)	511,99	+	A 29
86	f	69	Stg III (T4N1M0)	595,119576	+	A47
87	f	77	Stg III (T4N1M0)	3213,929626	+	A46
88	f	70	Stg III (T3N2M0)	875,972364	+	R14 A43
89	f	67	Stg III (T3N2M0)	350,169951	+	A41
90	m	56	Stg III (T3N2M0)	240,406793	+	A40
91	m	64	Stg IV (TxNxM1)	637,286342	+	M0 R29 +29
92	m	45	Stg IV (T3N0M1)	970,278044	+	M0 A47
93	m	37	Stg IV (T3N0M1)	256,704197	+	M0 M10 +27
94	m	55	Stg IV (T1N1M1)	99,392811	-	M0 A25
95	f	61	Stg IV (T3N1M1)	695,887341	+	M0 R15
96	f	72	Stg IV (T3N1M1)	192,70291	-	M0 R7 A23
97	m		Stg IV (T3N1M1)	94,871724	-	M0 R29 R37 A43
98	m	67	Stg IV (T4N1M1)	2985,066551	+	M0 R26 A43
99	f	72	Stg IV (T4N1M1)	94,929794	-	M0 +28
100	m	75	Stg IV (T3N2M1)	125,844697	-	M0 R6 +26
101	f	60	Stg IV (T4N1M1)	319,54	+	M0 R14 M16 A24
102	f	65	Stg IV (T4N2M1)	303,44	+	M 0 +15
103	m	74	Stg IV (T4N2M1)	941,9	+	M 0 +8
104	f	66	Stg IV (metas [b])	962,58	+	M 0 +6
105	m	66	Stg IV (metas)	109,39	-	M 0 R 10 +17
106	m	57	Stg IV (metas)	479,18	+	M 0 +8

a = staging and category of colon carcinoma according to TNM classifications. UICC, tumor, nodes, metastases.
T1-T4, invasive tumors (T1, submucosa; T2, tunica muscularis; T3, subserosa; T4, peritoneum or other organs).
N0, no malignant regional lymphnodes; N1, 1-3 regional lymphnodes metastases; N2, >4 regional lymphnode metastases.
M0, no distant metastases; M1, distant metastasis;
Stage I (T1-2, N0, M0), Stage II (T3-4, N0, M0), Stage III (T1-4, N1-2, M0), Stage IV (any T, any N, M1).
b = liver metastasis of colon carcinoma. m: male; f: female. OD= optical density (arbitrary units);
+, - = positive and negative reactivity using 237.2 OD cut-off.
A = alive; M= metastasis; R= relapse; += dead.

Supplemental Materials Table S2

Demographic and clinical features of the Pancreas cancer patients

Ptz #	Gender	Age	Stage (category)[b]	anti-ADAM 10 reactivity	
				normalized OD	237.2 OD cut-off
1	m	80	Stg I (T2N0M0)	227,6565312	-
2	m	49	Stg II (T3N0M0)	290,006681	+
3	m	61	Stg II (T3N0M0)	2941,625288	+
4	m	65	Stg II (T3N0M0)	268,6080692	+
5	m	55	Stg II (T3N0M0)	319,1174562	+
6	m	66	Stg II (T2N0M0)	2032,10902	+
7	m	65	Stg II (T3N0M0)	240,1435598	+
8	f	54	Stg II (T3N0M0)	1972,322728	+
9	f	75	Stg II (T3N0M0)	305,1569973	+
10	m	62	Stg II (T3N0MX)	129,6137602	-
11	m	71	Stg II (T3N0M0)	139,7745274	-
12	f	64	StgII(T3N0M0)	75,86736809	-
13	f	50	StgII (T3N0M0)	448,8767779	+
14	f	77	Stg III (T2N1M0)	430,6587305	+
15	m	66	Stg III (T3N1MX)	1023,285501	+
16	m	60	Stg III (T3N1MX)	273,012504	+
17	m	66	Stg III (T3N1M0)	1131,161439	+
18	f	80	Stg III (T3N1M0)	1309,252755	+
19	m	77	Stg III (T3N1M0)	156,7990702	-
20	m	65	Stg III (Npositivo)	1445,984434	+
21	f	53	Stg III (T3N1M0)	341,6848165	+
22	m	64	Stg III (T3N1M0)	544,3897833	+
23	f	69	Stg III (T3N1M0)	313,4547039	+
24	f	59	Stg III (T3N1M0)	121,6335325	-
25	f	77	Stg III (T3N1M0)	582,5253811	+
26	f	72	Stg III (T3N1M0)	385,2381419	+
27	m	84	Stg III (Npositivo)	80,17356146	-
28	f	64	Stg III (T3N1M0)	122,7571674	-
29	m	79	Stg III (T3N1M0)	267,215188	+
30	f	74	Stg III (T3N1M0)	217,1359626	-
31	m	80	Stg III (T3N1M0)	1561,809321	+
32	f	66	Stg III (T4N1M0)	190,1762314	-
33	f	74	Stg III (T2N0M1)	336,5519985	+
34	m	75	StgIII(T4N1M0)	46,05253594	-
35	f	72	StgIII(T4N1)	2124,287794	+
36	f	46	StgIII(T4N1)	3058,137141	+
37	m	50	StgIII(T4N1)	52,02744853	-
38	m	74	Stg IV (T4NXM1)	409,4999082	+
39	m	74	Stg IV (M1)	345,3794828	+
40	f	65	Stg IV (M1)	10471,13735	+
41	f	72	Stg IV (T4M1)	1695,106047	+
42	m	69	Stg IV (T2N0M1)	348,9650761	+
43	f	51	Stg X (T4NXMX)	265,700557	+

a = staging and category of colon carcinoma according to TNM classifications. UICC, tumor, nodes, metastases.
T1-T4, invasive tumors (T1, submucosa; T2, tunica muscularis; T3, subserosa; T4, peritoneum or other organs).
N0, no malignant regional lymphnodes; N1, 1-3 regional lymphnodes metastases; N2, >4 regional lymphnode metastases.
M0, no distant metastases; M1, distant metastasis;
Stage I (T1-2, N0, M0), Stage II (T3-4, N0, M0), Stage III (T1-4, N1-2, M0), Stage IV (any T, any N, M1).
b = liver metastasis of colon carcinoma. m: male; f: female. OD= optical density (arbitrary units);
+, - = positive and negative reactivity using 237.2 OD cut-off.

Supplemental Materials Table S3

Demographic and clinical features of the Breast cancer patients

Ptz #	Gender	Age	Stage (category)[b]	anti-ADAM 10 reactivity	
				normalized OD	237.2 OD cut-off
1	f	41	T1N0G2M0	106,66	-
2	f	41	T1bN0G3M0	106,80	-
3	f	48	T2N0G3M0	114,63	-
4	f	50	TisN0G2M0	336,46	+
5	f	47	T1bN0G2M0	148,25	-
6	f	61	T1cN0G2M0	128,19	-
7	f	51	T2N0G3M0	402,57	+
8	f	65	T1cN0G2M0	461,61	+
9	f	66	TisN0G2M0	35,86	-
10	f	68	T1cN0G1M0	345,60	+
11	f	71	T1cN1aG3M0	211,10	-
12	f	51	T1cN1G1M0	396,88	+
13	f	50	T4NXG1M0	336,67	+
14	f	67	T1cN0g2M0	466,06	+
15	f	35	T2 N0	112,96	-
16	f	29	T2 N1	172,04	-
17	f	48	T1 N0	471,54	+
18	f	59	T1 N0	387,47	+
19	f	43	T3 N1	319,02	+
20	f	42	T1 N1	428,27	+
21	f	42	T1 N0	302,12	+
22	f	64	T1 N0	540,44	+
23	f	58	T1 N0	1199,61	+
24	f	36	T1 N0	1174,86	+
25	f	69	T1 N0	370,80	+
26	f	62	T1 N0	645,55	+
27	f	46	T2 N0	706,35	+
28	f	40	T2 N0	459,58	+
29	f	52	T1 N0	706,17	+
30	f	62	T1 N2	513,05	+
31	f	50	T2 N0	274,06	+
32	f	39	T2 N0	663,65	+
33	f	44	T1 N1	232,55	-
34	f	46	T3 N1	194,63	-
35	f	60	T1 N1	1390,39	+

a = staging and category of colon carcinoma according to TNM classifications. UICC, tumor, nodes, metastases.
T1-T4, invasive tumors (T1, submucosa; T2, tunica muscularis; T3, subserosa; T4, peritoneum or other organs).
N0, no malignant regional lymphnodes; N1, 1-3 regional lymphnodes metastases; N2, >4 regional lymphnode metastases.
M0, no distant metastases; M1, distant metastasis;
Stage I (T1-2, N0, M0), Stage II (T3-4, N0, M0), Stage III (T1-4, N1-2, M0), Stage IV (any T, any N, M1).
b = liver metastasis of colon carcinoma. m: male; f: female. OD= optical density (arbitrary units);
+, - = positive and negative reactivity using 237.2 OD cut-off.

Supplemental Materials Table S4

Demographic and clinical features of the B-cell chronic lymphocytic leukemia patients

			anti-ADAM 10 reactivity	
Ptz #	Gender	Age	normalized OD	237.2 OD cut-off
1	m	63	923,19	+
2	m	78	117,30	-
3	m	60	185,70	-
4	m	75	257,74	+
5	m	67	194,92	-
6	f	-	489,89	+
7	m	79	754,92	+
8	m	58	47,42	-
9	m	57	46,78	-
10	m	64	219,63	-
11	f	79	23,78	-
12	m	-	575,67	+
13	m	71	232,24	-
14	m	-	283,72	+
15	m	60	256,52	+
16	m	65	740,21	+
17	f	-	507,66	+
18	m	75	600,33	+
19	f	42	273,08	+
20	m	75	519,81	+
21	m	68	150,15	-
22	m	66	252,71	+
23	m	69	361,91	+
24	f	55	56,68	-
25	m	73	114,10	-
26	m	46	282,75	+
27	f	71	759,50	+
28	f	60	993,82	+
29	m	60	37,08	-
30	f	54	617,99	+
31	m	82	88,86	-
32	m	56	797,55	+
33	f	72	252,95	+
34	m	78	201,21	-
35	f	67	11,53	-
36	m	53	401,19	+
37	f	76	38,14	-
38	m	46	85,79	-
39	f	73	152,59	-
40	f	80	83,24	-
41	m	40	271,15	+
42	m	55	171,23	-
43	f	55	178,88	-
44	m	61	774,54	+
45	f	69	249,43	+
46	f	49	424,39	+
47	f	76	552,25	+
48	m	87	418,18	+
49	f	72	67,13	-
50	m	70	138,61	-
51	f	71	178,86	-
52	m	79	370,21	+
53	m	55	107,19	-

m: male; f: female. OD= optical density (arbitrary units);

+, - = positive and negative reactivity using 237.2 OD cut-off.

Supplemental Materials Table S5

Demographic and clinical features of the Multiple Myeloma patients

			anti-ADAM 10 reactivity	
Ptz #	Gender	Age	normalized OD	237.2 OD cut-off
1	f	61	352,04	+
2	f	61	814,84	+
3	m	68	858,03	+
4	m	72	542,32	+
5	f	67	351,51	+
6	f	67	385,60	+
7	f	78	133,79	-
8	f	66	813,58	+
9	f	72	38,60	-
10	f	58	732,18	+
11	f	73	60,00	-
12	m	61	299,16	+
13	m	61	126,71	-
14	f	70	11,46	-
15	m	51	118,42	-
16	m	52	111,48	-
17	f	73	125,78	-
18	m	67	168,84	-
19	f	68	442,64	+
20	f	73	43,27	-
21	m	80	71,02	-
22	m	40	108,82	-
23	f	66	40,06	-
24	m	74	440,41	+
25	f	77	242,86	+
26	f	72	22,86	-
27	f	70	22,15	-
28	f	64	262,85	+
29	f	66	415,11	+
30	m	73	94,17	-
31	m	87	40,59	-
32	f	58	565,01	+
33	m	45	1281,05	+
34	f	67	76,56	-
35	m	55	378,01	+
36	m	75	1527,50	+
37	f	40	186,86	-
38	m	64	307,43	+
39	m	77	26,56	-
40	f	81	10,88	-
41	f	68	20,03	-
42	m	84	426,79	+
43	m	66	56,43	-
44	f	73	155,96	-
45	m	58	329,76	+
46	m	52	88,70	-

m: male; f: female. OD= optical density (arbitrary units);

+, - = positive and negative reactivity using 237.2 OD cut-off.

Supplemental Materials Table S6

Demographic and clinical features of the Control subjects

				anti-ADAM 10 reactivity	
Subject #	Gender	Age	Clinical status	normalized OD	237.2 OD cut-off
1	f	79	inguinal hernia	2,48	-
2	f	59	endometrial polyp	3,13	-
3	f	68	hepatic cyst	365,42	+
4	m	44	healthy	39,74	-
5	m	45	healthy	39,74	-
6	f	61	diverticulosis	261,29	+
7	f	73	cataract	80,05	-
8	m	71	cataract	25,93	-
9	f	76	rectocele	78,27	-
10	f	58	hyperparathyroidism	110,31	-
11	m	69	cirrhosisr	103,69	-
12	m	86	diabetes	420,81	+
13	m	69	rectal prolapse	199,16	-
14	m	73	inguinal hernia	323,74	+
15	m	81	pulmonary embolism	125,02	-
16	f	43	healthy	177,42	-
17	f	76	diabetes	130,37	-
18	f	27	healthy	176,83	-
19	f	58	healthy	125,49	-
20	m	69	healthy	176,11	-
21	m	73	healthy	228,38	-
22	f	50	cervical ectropion	351,28	+
23	f	58	healthy	306,22	+
24	f	57	thyroid goiter	233,93	-
25	f	70	diverticulosis	233,29	-
26	m	79	healthy	407,87	+
27	m	81	healthy	473,58	+
28	f	73	healthy	312,29	+
29	f	64	bronchial asthma	1,6	-
30	f	67	healthy	442,75	+
31	m	77	inguinal hernia	186,24	-
32	f	26	healthy	477,16	+
33	m	91	hemorroiditis	27,47	-
34	f	62	healthy	558,21	+
35	f	73	diabetes	515,14	+
36	f	30	healthy	126,18	-
37	f	67	healthy	734	+
38	m	60	healthy	127,21	-
39	f	61	cholecystitis	553,93	+
40	m	74	healthy	202,49	-
41	m	57	healthy	888,54	+
42	m	76	healthy	262,20	+
43	f	65	healthy	58,78	-
44	m	82	healthy	91,88	-
45	m	53	healthy	618,80	+

46	m	76	healthy	353,64	+
47	m	66	healthy	183,68	-
48	f	77	healthy	220,25	-
49	m	76	healthy	85,67	-
50	m	67	healthy	86,21	-
51	m	52	healthy	528,48	+
52	m	69	healthy	827,93	+
53	m	72	healthy	968,50	+
54	f	88	healthy	226,19	-
55	f	70	healthy	169,61	-
56	m	67	healthy	60,88	-
57	f	67	healthy	303,60	+
58	m	72	healthy	228,13	-
59	m	61	healthy	662,79	+
60	f	51	healthy	3,43	-
61	f	59	healthy	202,44	-
62	f	53	healthy	39,32	-
63	f	60	healthy	144,45	-
64	f	75	healthy	160,31	-
65	f	57	healthy	195,97	-
66	f	76	healthy	173,15	-
67	f	91	healthy	204,18	-
68	f	57	healthy	446,88	+
69	f	52	healthy	566,61	+
70	f	63	healthy	3,75	-
71	f	92	healthy	221,09	-
72	f	55	healthy	42,94	-
73	f	68	healthy	157,77	-
74	f	89	healthy	175,08	-
75	f	78	healthy	214,03	-
76	m	45	healthy	431,76	+
77	m	32	healthy	102,86	-
78	f	68	healthy	533,68	+
79	f	51	healthy	150,10	-
80	f	55	healthy	552,64	+
81	m	45	healthy	108,71	-
82	f	48	healthy	370,80	+
83	f	44	healthy	489,91	+
84	f	69	healthy	112,27	-
85	m	45	healthy	84,39	-
86	m	54	healthy	615,50	+
87	f	70	healthy	332,42	+
88	f	34	healthy	290,05	+
89	m	49	healthy	137,49	-
90	f	59	healthy	104,45	-
91	m	71	healthy	159,56	-

m: male; f: female. OD= optical density (arbitrary units);
+, - = positive and negative reactivity using 237.2 OD cut-off.