Dipeptidyl-Peptidase IV Activity Is Correlated with Colorectal Cancer Prognosis

Gorka Larrinaga1,2,5,*, Itxaro Perez1,2,5, Begoña Sanz2,5, Maider Beitia1,2,5, Peio Errarte2,5, Ainhoa Fernández2,5, Lorena Blanco2,5, María C. Etxezarraga3,5, Javier Gil2,5, José I. López4,5
1 Department of Nursing I, School of Nursing, University of the Basque Country (UPV/EHU), Leioa, Bizkaia, Spain, 2 Department of Physiology, Faculty of Medicine and Dentistry, University of the Basque Country (UPV/EHU), Leioa, Bizkaia, Spain, 3 Department of Anatomic Pathology, Basurto University Hospital, University of the Basque Country (UPV/EHU), Bilbao, Bizkaia, Spain, 4 Department of Anatomic Pathology, Cruces University Hospital, University of the Basque Country (UPV/EHU), Barakaldo, Bizkaia, Spain, 5 BioCruces Health Research Institute, Barakaldo, Bizkaia, Spain

* gorka.larrinaga@ehu.es

Abstract

Background
Dipeptidyl-peptidase IV (EC 3.4.14.5) (DPPIV) is a serine peptidase involved in cell differentiation, adhesion, immune modulation and apoptosis, functions that control neoplastic transformation. Previous studies have demonstrated altered expression and activity of tissue and circulating DPPIV in several cancers and proposed its potential usefulness for early diagnosis in colorectal cancer (CRC).

Methods and principal findings
The activity and mRNA and protein expression of DPPIV was prospectively analyzed in adenocarcinomas, adenomas, uninvolved colorectal mucosa and plasma from 116 CRC patients by fluorimetric, quantitative RT-PCR and immunohistochemical methods. Results were correlated with the most important classic pathological data related to aggressiveness and with 5-year survival rates. Results showed that: 1) mRNA levels and activity of DPPIV increased in colorectal neoplasms (Kruskal-Wallis test, \( p < 0.01 \)); 2) Both adenomas and CRCs displayed positive cytoplasmic immunostaining with luminal membrane reinforcement; 3) Plasmatic DPPIV activity was lower in CRC patients than in healthy subjects (Mann-U test, \( p < 0.01 \)); 4) Plasmatic DPPIV activity was associated with worse overall and disease-free survivals (log-rank \( p < 0.01 \), Cox analysis \( p < 0.01 \)).

Conclusion/significance
1) Up-regulation of DPPIV in colorectal tumors suggests a role for this enzyme in the neoplastic transformation of colorectal tissues. This finding opens the possibility for new therapeutic targets in these patients. 2) Plasmatic DPPIV is an independent prognostic factor in survival of CRC patients. The determination of DPPIV activity levels in the plasma may be a
safe, minimally invasive and inexpensive way to define the aggressiveness of CRC in daily practice.

**Introduction**

CRC is the third commonest malignancy in males and females in the United States, with more than 136,500 estimated new cases and more than 50,000 estimated deaths in 2014 [1]. Europe and other developed regions show similar rates of incidence and mortality [2]. Traditionally related to dietary habits [3], its frequency has steadily increased in the last decades in Western countries as a result of modern style of life to become a health problem of major concern. Huge resources are being invested in prevention and early diagnosis of this disease. Population-based screening campaigns try to discover as much early tumors and precursor lesions as possible, aiming to decrease the incidence of the disease, to simplify the clinical management of patients once the lesion develops, and to improve survival.

From a pathological perspective, adenomatous lesions in the large bowel are fully accepted precursors of CRC [4,5] and the adenoma-carcinoma sequence still provides a solid model for research on carcinogenesis [6]. However, a large number of cellular metabolic processes still largely unknown are involved in the origin and development of neoplastic processes [7].

Peptidases play a key role in carcinogenesis in several ways, for instance regulating bioactive peptides that are crucial in neoplastic growth and immune response, degrading the extracellular matrix, acting as adhesion molecules or participating in intracellular signalling [8]. Besides, some studies have demonstrated that the activity and expression of these enzymes vary in different tumors depending on the several pathological parameters like histological Grade, Stage, and patient survival [8-12]. For this reason, peptidases are useful tools in the development of clinical strategies for treatment and follow up of cancer patients.

Dipeptidyl-peptidase IV (EC 3.4.14.5) (DPPIV), also known as cluster differentiation antigen CD26, is a serine peptidase expressed on a variety of cells including epithelial, endothelial and lymphocytes [13]. As other membrane-bound glycoproteins, it also can be released in active form to body fluids such as plasma, serum and urine [14]. DPPIV cleaves N-terminal dipeptides from selected bioactive peptides including some cytokines, chemokines and neuropeptides, leading to their inactivation and/or degradation. Independent of its enzymatic activity, DPPIV interacts with extracellular matrix (ECM) components including collagen and fibronectin, thus regulating cell-cell and cell-ECM interactions, and with several receptors and proteases that leads to the secretion of matrix metalloproteases (MMPs). Through these multiple functions, DPPIV regulates diverse biological processes including cell differentiation, adhesion, immune modulation and apoptosis, functions that control neoplastic transformation [13].

Many studies have described that DPPIV expression and activity is significantly altered in several solid tumor tissues and that these changes are associated with tumor grade, stage and patients 5-year survival, which point to this enzyme as a potential diagnostic tool and therapeutic target [12,13,15]. Moreover, it has been demonstrated that blood DPPIV levels are significantly lower in cancer patients than in healthy individuals, a finding that has become of great interest in the development of additional tumor markers [14,16-19].

Regarding CRC, two recent studies described that higher immunohistochemical expression of DPPIV in CRC tissues is correlated with metastases and worse overall survival of CRC patients [20] and that circulating DPPIV levels are correlated with distant recurrence of CRC [21]. We and other groups also described that the expression and activity profile of other serine
peptidases related with DPPIV vary throughout the adenoma-adenocarcinoma sequence and that are independently correlated with survival of CRC patients [22–26]. All these accumulated evidences point to the analyses of serine peptidases such as DPPIV as promising tools in the design of new diagnostic/prognostic biomarkers and therapeutic targets in CRC [14,20–26].

In this context, the objective of the present work was to study in a prospective way the metabolic and expression profiles of DPPIV in patients with CRC analyzing tissue from the adenoma-adenocarcinoma sequence and plasma samples, and to correlate the obtained results with classic histopathological parameters for tumor prognosis and survival.

Materials & Methods

The authors declare that all experiments carried out in this study comply with current Spanish and European Union legal regulations. Samples and data from patients included in this study were provided by the Basque Biobank for Research-OEHUN (www.biobancovasco.org). All patients were informed about the potential use for research of their surgically resected tissues, and accepted this eventuality by signing a specific document approved by the Ethical and Scientific Committees of the Basque Country Public Health System (Osakidetza) (CEIC 11/51).

Patients

A total of 116 patients with CRC were prospectively included in the study. All patients received partial colectomies. The topographic distribution was 41 right sided CRCs, 51 left sided and 24 from the rectum. 7 patients received preoperative therapy, 49 patients received chemotherapy or chemo-radiotherapy after resection, and 60 patients did not receive any therapy.

In 20 patients, both adenomatous and adenocarcinomatous polyps were diagnosed. 13 adenomas were tubular, 6 were tubulovillous adenomas, and 1 villous adenoma. In addition, 16 showed mild to moderate dysplasia and 4 showed high grade dysplasia. The average size was 2.1 cm (with a range of 0.6–4cm). These 20 cases were used to analyze the DPPIV activity and mRNA/protein expression throughout the normal mucosa-adenoma-adenocarcinoma sequence.

The American Joint Committee on Cancer system (AJCC, 7th edition) [27] has been applied to assign Stage and Grade. Follow-up was closed by June 30, 2014. At that time, 43 patients (37%) had died of disease. Mean follow-up was 53 months (range 4–88).

Plasma was also collected preoperatively and analyzed in 98 of these patients. Plasma from 72 healthy volunteers with no clinical history of neoplastic diseases was used as control sample.

Clinical data included in the study were retrieved from the patient clinical records and are summarized in Table 1.

Tissue Specimens

Surgical resections were submitted in fresh to the Pathology Lab within a period of 30 minutes after removal. The material for this study comes from the excess tissue of pathological diagnosis. Handling of specimens was performed following conventional protocols for the management of surgical resections of colon and rectum [28]. Tumor characteristics were recognized on gross examination and selected fragments of tumor and non tumor tissues were frozen in isopentane and stored at -80°C. Routine procedures in the Pathology Lab included formalin fixation of the surgical specimen and paraffin embedding of the tissue fragments selected for histopathological examination. Histological slides were stained with hematoxylin-eosin. Besides, peripheral venous blood samples from 98 of these patients were obtained prior to surgery and centrifuged at 1500 rpm during 15 minutes. The obtained plasma was also stored at -80°C. Enzyme assay was been performed in plasma obtained from 72 healthy volunteers (matched by sex and age).
Sample preparation

Explanted tumor samples were homogenized in 10 mM Tris-HCl buffer at pH 7.4, for 30 seconds at 800 rpm using a Heidolph PZR 50 Selecta homogenizer, and ultracentrifuged in a Centrifkon T-2070 Kontron Instruments apparatus at 100,000 g for 35 minutes. To avoid contamination with soluble enzymes, the resulting pellets were washed three times by suspension in 10 mM Tris-HCl buffer at pH 7.4. Pellets were then homogenized in 10 mM Tris-HCl buffer at pH 7.4, and centrifuged at low speed (1500 g) for 3 min to purify the samples. The supernatants thus obtained were used to determine DPPIV activity and protein concentrations. All the aforementioned steps were carried out at 4°C.

DPPIV activity measurements

DPPIV activity was measured in triplicate by using H-Gly-Pro-β-naphthylamide as substrate, following a modified version of the method of Alponti et al. [29]. The assay is based on the

Table 1. Clinical and pathologic characteristics of patients with colorectal cancer (CRC), both for CRC tissue and plasmatic samples.

| Variables            | CRC tissue n = 116 | Plasma n = 98 |
|----------------------|--------------------|---------------|
| **Age**              |                    |               |
| Average              | 70                 | 68            |
| Range                | 32–89              | 32–85         |
| **Sex**              |                    |               |
| Male                 | 72                 | 63            |
| Female               | 44                 | 35            |
| **Grade**            |                    |               |
| Low (G1–G2)          | 95                 | 81            |
| High (G3–G4)         | 21                 | 17            |
| **Local invasion**   |                    |               |
| Low (T1–T2)          | 18                 | 25            |
| High (T3–T4)         | 98                 | 73            |
| **Nodal invasion**   |                    |               |
| No                   | 76                 | 73            |
| Yes                  | 40                 | 25            |
| **Distant Metastases**|                   |               |
| No                   | 106                | 89            |
| Yes                  | 10                 | 9             |
| **Lymphatic invasion**|                   |               |
| No                   | 103                | 84            |
| Yes                  | 13                 | 14            |
| **Vascular invasion**|                   |               |
| No                   | 95                 | 80            |
| Yes                  | 21                 | 18            |
| **Perineural invasion**|                 |               |
| No                   | 110                | 94            |
| Yes                  | 6                  | 4             |
| **Grouped stage**    |                    |               |
| Low (0-IIC)          | 71                 | 70            |
| High (IIIA-IV)       | 45                 | 28            |

doi:10.1371/journal.pone.0119436.t001
fluorescence of products generated from the hydrolysis of the substrate by the enzyme. Reactions were initiated by adding 30–50 μL of sample to 1 mL of the appropriate incubation mixture (50 mM Tris-HCl buffer, pH 7.4, and 0.2 mM aminoacyl-β-naphthylamide). After 30 min incubation at 37°C, 1 mL of 0.1 M sodium acetate buffer (pH 4.2) was added to the mixture to terminate the reaction. The released product was determined by measuring the fluorescent intensity with a Shimadzu RF-540 Spectrofluorophotometer (the excitation and emission wavelengths were 345 and 412 nm, respectively). Blanks were used to determine background fluorescence. The relative fluorescence was converted into picomoles of product using a standard curve constructed with increasing concentrations of β-naphthylamine.

To verify that the formation of β-naphthylamine (β-NA) was due to the action of DPPIV and not due to other enzymes, we performed inhibition assays in CRC tissue and plasma with a specific inhibitor of the enzyme (diprotin A, 0.2mM). The releasing of β-NA was mainly inhibited in colorectal tissues (82%) and in plasma samples (86%).

Protein concentration was measured in triplicate by the Bradford method [30], using BSA (1 mg/mL) as the calibrator. Results from the CRC tissues and from plasma samples were recorded as units of peptidase per milligram of protein (UP/mg prot) and per liter of plasma (UP/L), respectively. One unit of peptidase activity (UP) is the amount of enzyme required to release one pmol of β-naphthylamine per minute. Fluorogenic assays were linear with respect to hydrolysis time and protein content.

**Immunohistochemistry**

Formalin-fixed and paraffin-embedded tissue from 20 CRCs, adenomatous polyps and the uninvolved surrounding mucosa were immunostained with a monoclonal antibody specific for DPPIV/CD26 (Novus Biologicals NB 100–59021, rabbit anti-human, working dilution 1:250). The immunostaining process was performed following routine methods in an automatic immunostainer (Dako Autostainer Plus). In short, endogenous peroxidase activity was blocked by incubating the slides in 3% hydrogen peroxide in absolute methanol for 10 minutes. Antigen retrieval was carried out in citrate buffer (10 mM, pH = 6) for 15 minutes at 100°C in a microwave oven. The primary antibody was applied for 1 hour at room temperature. A subsequent reaction was performed with secondary antibodies and biotin-free HRP enzyme labeled polymer of the EnVision-Flex detection system (Dako, Carpinteria, CA). Nonspecific IgG was used as a negative control. A positive reaction was visualized with diaminobenzydine solution followed by counterstaining with haematoxylin.

**Real-time quantitative RT-PCR Analysis**

The expression of DPP4, the gene that encode DPPIV, was analysed in tissue from 20 CRCs, adenomatous polyps and the uninvolved surrounding mucosa. To avoid degradation, tissue sections were immersed in RNAlater immediately after surgery and stored at -80°C until processed. Total RNA was extracted from 20 mg of tissue using TriReagent (Sigma, St. Louis, MO). The RNA samples were then incubated with RNase-free DNase I and RNasin (Promega, Madison, WI) to remove residual genomic DNA. First-strand cDNA was synthesized from 5 μg of total RNA of each human sample using First-strand cDNA synthesis kit (Roche, Mannheim, Germany). The resulting cDNA samples were amplified by PCR with specific oligonucleotide primer pairs designed with the analysis software Primer 3 and synthesised by Sigma-Genosys (Cambridge, UK). Based on previous experiments on human renal cell carcinoma [15], CRC [12] and other human tissues [31] we chose glyceraldehyde-3-phosphate dehydrogenase (GAPDH), polymerase (RNA) II (DNA-directed) polypeptide A (POLR2A), hypoxanthine phosphoribosyltransferase 1 (HPRT1), β-actin (ACTB), succinate dehydrogenase complex,
subunit A (SDHA), TATA box binding protein (TBP) and peptidylprolyl isomerase A (PPIA) as endogenous reference genes. The sequence of the primers used to amplify DPP4 and the seven housekeeping genes are shown in Table 2.

The expression of DPP4 and the housekeeping genes was quantified in all cDNAs by real-time PCR using the iCycler iQ real-time detection apparatus (BioRad Laboratories, Hercules, CA, USA). Experiments were carried out essentially as described previously [12,15,32]. Dilutions of the cDNA template were prepared from each tissue and amplified in triplicate using SensiFAST SYBR Mix (Bioline Ltd., London, UK). Three negative controls (with no template, no reverse transcriptase and no RNA in the reverse transcriptase reaction) were also included in each plate to detect any possible contamination. After a hot start (10 min at 94°C), the parameters used for PCR amplification were: 10 s at 94°C, 20 s at 60°C and 30 s at 72°C, for 50 cycles.

Real-time PCR data were expressed as the fold change of the target gene expression relative to the geometric mean (g.m.) mRNA expression of the housekeeping genes in each sample, as described by Vandesompele et al. [33]. The fold change in gene expression was calculated by the formula: $2^{-\Delta\Delta CT}$, where $CT$ is the threshold cycle, calculated by the iCycler software, $\Delta CT = (CT_{target\text{ gene}} - CT_{g.m.\text{ reference\ genes}})$ and $\Delta\Delta CT = (\Delta CT_{\text{test sample}} - \Delta CT_{\text{control sample}})$.

Samples from the same patient were always measured in the same analytical run to exclude between-run variations. PCR data obtained in one of the normal CRC samples were arbitrarily chosen as control, and this sample was included in all PCR experiments to correct for possible interassay variations.

Statistical analysis

Kolmogorov-Smirnov and Shapiro-Wilk tests were applied to data obtained from tissue and plasma samples respectively to know if the numbers followed or not a normal distribution. Based on this information (p<0.05), DPPIV activity in tissue and plasma was analyzed with non parametric probes: Mann-Whitney test and Kruskal-Wallis tests were used to detect differences between two or three groups, respectively.

Finally, Kaplan-Meier curves and log-rank test were performed to evaluate the association between DPPIV activity and 5-year survival, comparing groups created by cut-off points based

| Peptidase Gene name | Peptidase Gene symbol | Forward primer | Reverse primer | Amplicon size (bp) |
|---------------------|-----------------------|----------------|----------------|-------------------|
| Peptidase Dipeptidylpeptidase IV | DPP4 | 5'-AGTGGCGTGTCAAGGTTGG-3' | 5'-CAAGGTTGTGCTTCTGGAGTGG-3' | 112 |
| Housekeeping Gene name | GAPDH | 5'-CAATGCTCTGACACCACT-3' | 5'-CTCTGCTACACCTTCTTG-3' | 350 |
| Glyceraldehyde-3-phosphate dehydrogenase | POLR2A | 5'-ACATCTACGTCTGCTTACTCC-3' | 5'-GTCTGTTGTCTCAGGATCTG-3' | 268 |
| Polypeptide (RNA) II (DNA directed) | HPRT1 | 5'-GCCAGAGTGGTGAGTGG-3' | 5'-GCTTTGATATTGGCTTCC-3' | 130 |
| Hypoxanthine phosphoribosyltransferase 1 | ACTB | 5'-CTCTGCTCAAGCCTTCTTG-3' | 5'-GCTTTGATATTGGCTTCC-3' | 142 |
| Beta-actin | GAPDH | 5'-CTCTGCTCAAGCCTTCTTG-3' | 5'-GCTTTGATATTGGCTTCC-3' | 139 |
| Succinate dehydrogenase complex, subunit A | TBP | 5'-GGATAAGAGCGCCAGAACCC-3' | 5'-TTAGCTGGAAACCCCAACTTCTG-3' | 139 |
| TATA box binding protein | PPIA | 5'-GGTCCCAAAGACAGCAAGAAA-3' | 5'-TCACCACCTGACACATAACC-3' | 114 |

Table 2. Sequence of forward (F) and reverse primers (R) of indicated target genes and the size expected for each PCR-amplified product.

Primer sequences for the assayed housekeeping genes are also shown.

doi:10.1371/journal.pone.0119436.t002
on receiver-operating characteristic (ROC) curves. A Cox regression model was used to test the independent effects of clinical and pathological variables and DPPIV activity on survival. SPSS® 21.0 software was used for the statistical analysis.

Results

DPPIV activity and expression in colorectal tissues from CRC patients

When DPPIV activity was analyzed throughout the colorectal adenoma-adenocarcinoma sequence, higher activity was found in CRCs and adenomas than in the adjacent uninvolved mucosa (Kruskal-Wallis test, p = 0.015). DPP4 mRNA expression showed a similar pattern, with higher levels in both neoplasms than in the non tumor tissue (Kruskal-Wallis test, p = 0.001). These results are described in Table 3.

When data were stratified by topographic distribution of CRCs, we did not found any significant difference (Kruskal-Wallis test, p = 0.965). Tissue DPPIV activity was also similar between patients who received preoperative treatment, postoperative therapy and patients who did not received any therapy after resection (Kruskal-Wallis test, p = 0.842).

Fig. 1 shows the immunohistochemical expression of DPPIV in colonic adenocarcinoma, adenoma and normal adjacent mucosa. Normal mucosa did not express DPPIV immunostaining. However, both adenomas (tubular, tubulovillous and villous) and CRCs displayed positive cytoplasmic immunostaining with luminal membrane reinforcement. The inflammatory component in all cases was also positive.

Tissue DPPIV activity according to 5-year survival and to pathologic characteristics

To determine the best cut-off values for overall survival (OS) (Fig. 2A) and disease-free survival analyses (DFS) (Fig. 2B), ROC curves were performed. For tissue DPPIV activity, 2600 UP/mg protein cut-off point showed the most optimal sensitivity and specificity ratios (Se = 47% and Sp = 55% for OS, and Se = 48% and Sp = 55% for DFS) (Fig. 2A and 2B).

| (A) DPPIV activity | UP/ mg prot (Media ± S.E) | Mann-Whitney (p =) |
|------------------|--------------------------|-------------------|
| Adenocarcinoma   | 2713 ± 627               | 0.806a            |
| Adenoma          | 2982 ± 659               | 0.007b            |
| Uninvolved mucosa| 990 ± 139                | 0.027c            |

| (B) DPP4         | Relative Units           | Mann-Whitney (p =) |
|------------------|--------------------------|-------------------|
| Adenocarcinoma   | 241 ± 66                 | 0.594a            |
| Adenoma          | 199 ± 35                 | 0.021b            |
| Uninvolved mucosa| 43 ± 14                  | 0.001c            |

(A) DPPIV/CD26 activity in colorectal tissues from CRC patients. Values are means ± SE of peptidase activity recorded as pmol of units of peptidase (UP) per milligram of protein. Differences were statistically significant (Kruskal-wallis test, p = 0.015). (B) DPP4 gene expression in colorectal tissues from CRC patients. qRT-PCR data for each analyzed sample are recorded as relative units (see text for details). Differences were statistically significant (Kruskal-wallis test, p = 0.001).

(a) Adenocarcinoma vs adenoma;
(b) Adenocarcinoma vs uninvolved mucosa;
(c) Adenoma vs uninvolved mucosa.

doi:10.1371/journal.pone.0119436.t003
Kaplan-Meier curves and log-rank test showed that five-year OS and DFS of patients with CRC was not correlated with tissue DPPIV activity (log-rank test, p = 0.8 for OS; and p = 0.67 for DFS) (Fig. 3A and 3B, respectively).

Similarly, the stratification of DPPIV activity by several pathologic variables did not throw any statistically significant result (Table 4).

**DPPIV activity in plasma samples from CRC patients**

In plasma from CRC patients DPPIV activity was significantly lower than in healthy individuals (175 ± 7.2 UP/L vs. 218 ± 10.1 UP/L, Mann-Whitney test p < 0.01) (Fig. 4). However the stratification of data according to pathological variables did not yield any significant result (Table 5).

Kaplan-Meier curves and log-rank test showed that five-year OS and DFS of patients with CRC was not correlated with tissue DPPIV activity (log-rank test, p = 0.8 for OS; and p = 0.67 for DFS) (Fig. 3A and 3B, respectively).

Similarly, the stratification of DPPIV activity by several pathologic variables did not throw any statistically significant result (Table 4).

**DPPIV activity in plasma samples from CRC patients**

In plasma from CRC patients DPPIV activity was significantly lower than in healthy individuals (175 ± 7.2 UP/L vs. 218 ± 10.1 UP/L, Mann-Whitney test p < 0.01) (Fig. 4). However the stratification of data according to pathological variables did not yield any significant result (Table 5).
In plasma samples ROC curves were performed both for DPPIV activity and carcinoembryonic antigen (CEA) levels. For plasmatic DPPIV activity, 165 UP/L cut-off value showed the most optimal sensitivity and specificity ratios (Se = 64% and Sp = 63% for OS, and Se = 65% and Sp = 58% for DFS) (Fig. 5A and 5B). A cut-off value of 5 ng/mL for CEA, which is known to have a prognostic impact in CRC [34], showed lower sensitivity (44% for OS, and 39% for DFS) but higher specificity (68% for OS, and 64% for DFS) than DPPIV (Fig. 5A and 5B).

Fig 3. Kaplan-Meier curves for DPPIV activity in CRC tissues. Overall survival (A) and disease-free survival curves (B) of CRC patients according to their tumor DPPIV activity pattern. The number of patients at risk in each group at individual time points is also included.

doi:10.1371/journal.pone.0119436.g003
### Table 4. DPPIV/CD26 activity in CRC tissue according to pathologic characteristics.

| Variables               | UP/mg prot (Media ± S.E) | Mann-Whitney (p =) |
|-------------------------|---------------------------|--------------------|
| **Grade**               |                           |                    |
| Low (G1–G2)             | 3357 ± 408                | 0.648              |
| High (G3–G4)            | 2959 ± 673                |                    |
| **Local invasion**      |                           |                    |
| Low (T1–T2)             | 1750 ± 292                | 0.061              |
| High (T3–T4)            | 3506 ± 396                |                    |
| **Nodal invasion**      |                           |                    |
| No                      | 3221 ± 415                | 0.821              |
| Yes                     | 3376 ± 643                |                    |
| **Distant Metastases**  |                           |                    |
| No                      | 3082 ± 315                | 0.523              |
| Yes                     | 5295 ± 1212               |                    |
| **Lymphatic invasion**  |                           |                    |
| No                      | 3046 ± 298                | 0.742              |
| Yes                     | 5332 ± 1245               |                    |
| **Vascular invasion**   |                           |                    |
| No                      | 2935 ± 329                | 0.128              |
| Yes                     | 4856 ± 1217               |                    |
| **Perineural invasion** |                           |                    |
| No                      | 3225 ± 366                | 0.135              |
| Yes                     | 4317 ± 1239               |                    |
| **Grouped stage**       |                           |                    |
| Low (0-IIC)             | 3022 ± 375                | 0.532              |
| High (IIIA-IV)          | 3615 ± 651                |                    |

Values are means ± SE of peptidase activity recorded as pmol of units of peptidase (UP) per milligram of protein.

doi:10.1371/journal.pone.0119436.t004

---

**Plasmatic DPPIV activity**

![Plasmatic DPPIV activity](image)

**Fig 4. Plasmatic DPPIV activity in CRC patients and healthy subjects (controls).** Values are means ± SE of units of peptidase per liter of plasma (UP/L). (*) Student’s T test, p<0.01.

doi:10.1371/journal.pone.0119436.g004
Kaplan-Meier curves and log-rank test showed that both OS (Fig. 6) and DFS (Fig. 7) were inversely correlated with plasmatic DPPIV activity. Thus, when plasmatic DPPIV activity was higher than 165 UP/L the OS and DFS were significantly worse (log-rank p = 0.009 and = 0.018, respectively) (Figs. 6A and 7A).

On the other hand, multivariate analysis showed that plasmatic DPPIV activity (p = 0.001), histological grade (p = 0.02) and local invasion (p = 0.01) were independent factors influencing patient OS (Fig. 6B), and that plasmatic DPPIV activity (p = 0.005), vascular invasion (p = 0.02) and grouped stage (p = 0.02) were independent prognostic factors of DFS (Fig. 7B).

Table 5. DPPIV/CD26 activity in plasma from CRC patients according to pathologic characteristics.

| Variables          | UP/L (Media ± S.E) | Mann-Whitney (p =) |
|--------------------|--------------------|--------------------|
| **Grade**          |                    |                    |
| Low (G1–G2)        | 176 ± 11           | 0.053              |
| High (G3–G4)       | 124 ± 26           |                    |
| **Stage**          |                    |                    |
| Low (T1–T2)        | 200 ± 28           | 0.271              |
| High (T3–T4)       | 157 ± 11           |                    |
| **Nodal invasion** |                    |                    |
| No                 | 171 ± 13           | 0.633              |
| Yes                | 157 ± 21           |                    |
| **Distant Metastases** |                  |                    |
| No                 | 166 ± 11           | 0.939              |
| Yes                | 178 ± 42           |                    |
| **Lymphatic invasion** |                |                    |
| No                 | 166 ± 11           | 0.985              |
| Yes                | 170 ± 34           |                    |
| **Vascular invasion** |                  |                    |
| No                 | 169 ± 12           | 0.774              |
| Yes                | 158 ± 23           |                    |
| **Perineural invasion** |                |                    |
| No                 | 165 ± 11           | 0.352              |
| Yes                | 195 ± 29           |                    |
| **Grouped stage**  |                    |                    |
| Low (0–IIC)        | 171 ± 13           | 0.535              |
| High (IIIA–IV)     | 155 ± 19           |                    |

Values are means ± SE of units of peptidase per liter of plasma (UP/L).

doi:10.1371/journal.pone.0119436.t005

Kaplan-Meier curves and log-rank test showed that both OS (Fig. 6) and DFS (Fig. 7) were inversely correlated with plasmatic DPPIV activity. Thus, when plasmatic DPPIV activity was higher than 165 UP/L the OS and DFS were significantly worse (log-rank p = 0.009 and = 0.018, respectively) (Figs. 6A and 7A).

On the other hand, multivariate analysis showed that plasmatic DPPIV activity (p = 0.001), histological grade (p = 0.02) and local invasion (p = 0.01) were independent factors influencing patient OS (Fig. 6B), and that plasmatic DPPIV activity (p = 0.005), vascular invasion (p = 0.02) and grouped stage (p = 0.02) were independent prognostic factors of DFS (Fig. 7B).

**Discussion**

Malignant transformation from normal to cancerous tissue is associated with cell-surface glycoprotein modifications. These phenotypic changes may play a crucial role in oncogenesis and can be useful tumor markers in distinguishing malignant from benign tissues [8]. Regarding CRC, the adenoma-carcinoma sequence in the large bowel describes that the gradual progression from normal to dysplastic epithelium, and hence to carcinoma, is the result of the successive accumulation of genetic mutations that lead to altered levels of several glycoproteins, like some peptidases [6,8].

The first relevant result in our study was that DPPIV showed higher activity and mRNA levels in preneoplastic adenomatous lesions and in CRC when compared with the uninvolved
Fig 5. ROC curves for plasmatic DPPIV activity and CEA levels. Optimal sensitivity and specificity ratios were observed using the following cut-off value: 165 UP/L for plasmatic DPPIV activity both for OS (A) and DFS (B). A cut-off value of 5 ng/mL for CEA, showed lower sensitivity but higher specificity than DPPIV.

doi:10.1371/journal.pone.0119436.g005
surrounding mucosa, although it did not correlate with pathological variables and with 5-year survival of CRC patients. Previously, it was reported that the activity and expression of two other serine peptidases, fibroblast activation protein alpha (FAP) and prolyl endopeptidase (PEP), was higher in adenomas and in early stages of CRC respectively [22,24]. In addition, FAP expression correlated with worse survival [22,23] and PEP activity did it with a better overall and disease-free survival [25]. Although these divergent influences on CRC prognosis suggest different acting roles for these serine peptidases in this disease, the expression and activity increases of these peptidases in neoplastic and preneoplastic tissues should be taken into account when designing new therapeutic approaches for colorectal cancer.

Due to its variable expression on solid tumors and to its diverse biological functions, the exact role that DPPIV plays in cancer remains to be elucidated. It has been described that DPPIV may either promote or impede tumor development depending on the specific type of tumor or on the phase of development the tumor is [13]. This phenomenon of tumor specificity sets forth practical difficulties when applying peptidase inhibitors in cancer therapies, and this point is being actively investigated nowadays [35,36]. A new trend in this topic consists in the use of cytotoxic prodrugs enabled to be activated by specific peptidases only where these peptidases are more active or highly expressed, aiming to damage the neoplastic cell without

Fig 6. Overall survival (OS) of CRC patients according to their plasmatic DPPIV activity pattern. (A) Kaplan-Meier curve and log-rank test. (B) Multivariate analysis of clinicopathologic variables and plasmatic DPPIV activity in predicting OS. The number of patients at risk in each group at individual time points is also included.

doi:10.1371/journal.pone.0119436.g006
modifying the enzyme functionality [35,36]. Some investigators are working in prodrugs with FAP as a target [35]. Since its homologous DPPIV is also highly active in adenomas and CRC, we suggest that this serine peptidase could be the target of similar prodrugs.

Cell-surface glycoproteins can be released to the extracellular space, appear in diverse body fluids and have become useful serum or plasmatic biomarkers for helping in screening, diagnosis, staging, prognosis and monitoring cancer therapy. The most relevant example in the clinical following of CRC patients is the carcinoembryonic antigen (CEA) [34]. However, the analysis of DPPIV in the plasma or serum of these patients has proved to be a reliable method in the early detection of CRC, being complementary to the classic faecal occult blood exam and other serum biomarkers which are under clinical investigation nowadays [14,17–20].

Several authors [14,17,19] have described decreases in the circulating levels of DPPIV of CRC patients compared with healthy subjects. In addition, increases of this enzyme have been observed in the serum of metastatic vs. non metastatic patients [14,17,19,20]. These data were obtained by immunodetection, suggesting its potential usefulness for early diagnosis and clinical follow-up. We also detected that the activity of DPPIV measured by fluorimetric methods was significantly decreased in the plasma of CRC patients. By contrast, we found that this activity was not correlated with metastatic status or with any other pathological variable.
The high differences in the number of patients between the groups with and without metas-
tases could be on the origin of this partial discrepancy. Other causes should also be considered
because it has been described that several biological phenomena may influence the activity of
circulating DPPIV/CD26 without affecting the protein level. In this sense the presence of circu-
lating molecules that alter the activity of this enzyme has been reported [37,38]. Nazarian et al.
[38] have shown in a very recent study that metastatic prostate cancer patients presented de-
creased circulating DPPIV activity while protein levels were similar with respect to patients
with localized primary tumor. This finding pointed to a circulating small peptide as a possible
cause of this inhibition [38] and opens new perspectives for further studies to clarify the poten-
tial usefulness of the combined analysis of DPPIV activity and protein levels in the serum/
plasma of cancer patients.

Since plasmatic levels and activities of the PEP and FAP have also been correlated with the
survival of CRC patients [19,25], a main objective of our study was to analyze prospectively the
correlation among plasmatic activity of its homologous DPPIV and 5-year survival of the pa-
tients. Data showed that patients with higher plasmatic DPPIV activities had poorer overall and
disease-free survival. These data showed better sensitivity but worse specificity than those ob-
tained with CEA. The multivariate analysis demonstrated that DPPIV is an independent prog-
nostic factor influencing CRC patient survival. Therefore, there are convincing evidences that
the determination of circulating DPPIV may be helpful in the early diagnosis [14,17–19,21] and
also in the prognosis of CRC.

The origin of circulating DPPIV in patients with cancer is a controversial issue. Current hy-
potheses place its origin in the liver and the immune cells [14] and in tumor microenviron-
ment [8,14,19,38]. We performed immunohistochemical analysis throughout the adenoma-carcinoma
sequence to know the location of DPPIV in the colorectal tissues. Non neoplastic cells of colorec-
tal mucosa did not stain with DPPIV. By contrast, adenoma and CRC cells showed positive cyto-
plasmic immunostaining with luminal membrane reinforcement. This immunohistochemical
pattern agrees with the higher activity and mRNA levels we have also found in neoplasms. Addi-
tionally, we found immunostaining with DPPIV in the inflammatory cells of lamina propria in
normal and neoplastic mucosa. This finding could suggest that DPPIV is released from both im-
mune and neoplastic cells in CRCs.

In conclusion, the present study demonstrates that DPPIV is up-regulated in adenomatous
and CRC tissues when compared with the uninvolved mucosa. Plasmatic DPPIV activity is
lower than in healthy subjects and is independently associated with worse 5-year survival in
CRC patients. The DPPIV activity determination in the plasma is a safe, minimally invasive
and inexpensive method, and may be complementary to the determination of the CD26 pro-
tein levels CRC patients. New studies with higher number of patients, collecting the preopera-
tive and postoperative plasma samples at several time points [21], should be performed to
confirm the predictive value of these results at diagnostic time and during patients’ follow-up.

Acknowledgments

We wish to thank Arantza Pérez (UPV/EHU) for her technical contribution to this study and
to Professor Juan Bilbao (UPV/EHU) for his statistical support.

Author Contributions

Conceived and designed the experiments: GL IP LB JIL. Performed the experiments: IP BS MB
PE AF. Analyzed the data: GL IP JG JIL. Contributed reagents/materials/analysis tools: MCE
JIL. Wrote the paper: GL JG JIL.
References

1. Siegel R, Desantis C, Jemal A. Colorectal cancer statistics. CA Cancer J Clin. 2014; 64:104–117. doi: 10.3322/caac.21220 PMID: 24639052

2. Ferlay J, Steliarova-Foucher E, Lortet-Tieulent J, Rosso S, Coebergh JW, Comber H et al. Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012. Eur J Cancer 2013; 49: 1374–1403. doi: 10.1016/j.ejca.2012.12.027 PMID: 23485231

3. Vargas AJ, Thompson PA. Diet and nutrient factors in colorectal cancer risk. Nutr Clin Pract 2012; 27: 614–623. doi: 10.1177/0884536612454885 PMID: 22892274

4. Kim EC, Lance P. Colorectal polyps and their relationship to cancer. Gastroenterol Clin North Am. 1997; 26: 1–17. PMID: 919435

5. Jass JR. Classification of colorectal cancer based on correlation of clinical, morphological and molecular features. Histopathology 2007; 50: 113–130. PMID: 17204026

6. Leslie A, Carey FA, Pratt NR, Steele RJ. The colorectal adenoma-carcinoma sequence. Br J Surg. 2002; 89: 845–860. PMID: 12081733

7. De la Fuente IM. Metabolic dissipative structures. In: Systems Biology of Metabolic and Signaling Networks: Energy, Mass and Information Transfer. Aon MA, Saks V, Schlattner U, eds. Springer Series in Biophysics 16, Springer New York, 2014, pp 179–212.

8. Carl-McGrath S, Lendeckel U, Ebert M, Röcken C. Ectopeptidases in tumor biology: A review. Histol Histopathol 2006; 21: 1339–1353. PMID: 16977585

9. Hashida H, Takabayashi A, Kanai M, Adachi M, Kondo K, Kohno N, et al. Aminopeptidase N is involved in cell motility and angiogenesis: its clinical significance in human colon cancer. Gastroenterology 2002; 122: 376–386. PMID: 11832452

10. Murakami H, Yokoyama A, Kondo K, Nakanishi S, Kohno N, Miyake M. Circulating aminopeptidase N/CD13 is an independent prognostic factor in patients with non-small cell lung cancer. Clin Cancer Res 2005; 11: 8674–8679. PMID: 16361553

11. Larrinaga G, Blanco L, Sanz B, Perez I, Gil J, Unda M, et al. The impact of peptidase activity in clear cell renal cell carcinoma survival. Am J Physiol Renal Physiol 2012; 303: F1584–1591. doi: 10.1152/ajprenal.00477.2012 PMID: 23019229

12. Larrinaga G, Perez I, Ariz U, Sanz B, Beitia M, Errarte P, et al. Clinical impact of aspartyl aminopeptidase expression and activity in colorectal cancer. Transl Res 2013; 162: 297–308. doi: 10.1016/j.trsl.2013.07.010 PMID: 23948443

13. Havre PA, Abe M, Urasaki Y, Ohnuma K, Morimoto C, Dang NH. The role of CD26/dipeptidyl peptidase IV in cancer. Front Biosci 2008; 13: 1634–1645. PMID: 17981655

14. Cordero OJ, Salgado FJ, Nogueira M. On the origin of serum CD26 and its altered concentration in cancer patients. Cancer Immunol Immunother 2009; 58: 1723–1747. doi: 10.1007/s00262-009-0728-1 PMID: 19557413

15. Varona A, Blanco L, Perez I, Gil J, Irazusta J, López JL, et al. Expression and activity profiles of DPPIV/CD26 and NEP/CD10 glycoproteins in the human renal cancer are-type dependent. BMC Cancer 2010; 10: 193. doi: 10.1186/1471-2407-10-193 PMID: 20459800

16. Hundt S, Haug U, Brenner H. Blood markers for early detection of colorectal cancer: a systematic review. Cancer Epidemiol Biomarkers Prev 2007; 16: 1935–1953. PMID: 17932341

17. Cordero OJ, Ayude D, Nogueira M, Rodríguez-Berrocal FJ, Páez de la Cadena M. Preoperative serum CD26 levels: diagnostic efficiency and predictive value for colorectal cancer. Br J Cancer 2000; 80: 1139–1146.

18. De Chiara L, Rodríguez-Piñeiro AM, Rodríguez-Berrocal FJ, Cordero OJ, Martínez-Ares D, Páez de la Cadena M. Serum CD26 is related to histopathological polyp traits and behaves as a marker for colorectal cancer and advanced adenomas. BMC Cancer 2010; 10: 333. doi: 10.1186/1471-2407-10-333 PMID: 20584285

19. Javidroozi M, Zucker S, Chen WT. Plasma seprase and DPP4 levels as markers of disease and prognosis in cancer. Dis Markers 2012; 32: 309–320. doi: 10.3233/DMA-2011-0889 PMID: 22674411

20. Lam CS, Cheung AH, Wong SK, Wan TM, Ng L, Chow AK, et al. Prognostic significance of CD26 in patients with colorectal cancer. PLoS One 2014; 9: e98582. doi: 10.1371/journal.pone.0098582 PMID: 24870408

21. De Chiara L, Rodríguez-Piñeiro AM, Cordero OJ, Vázquez-Tuñas L, Ayude D, Rodríguez-Berrocal FJ et al. Postoperative serum levels of sCD26 for surveillance in colorectal cancer patients. PLoS One 2014; 9: e107470. doi: 10.1371/journal.pone.0107470 PMID: 25210927
22. Henry LR, Lee HO, Lee JS, Klein-Szanto A, Watts P, Ross EA, et al. Clinical implications of fibroblast activation protein in patients with colon cancer. Clin Cancer Res. 2007; 13: 1736–1741. PMID: 17363526

23. Wikberg ML, Edin S, Lundberg IV, Van Guelpen B, Dahlin AM, Rutegard J, et al. High intratumoral expression of fibroblast activation protein (FAP) in colon cancer is associated with poorer patient prognosis. Tumor Biol 2013; 34: 1013–1020. doi: 10.1007/s13277-012-0638-2 PMID: 23328994

24. Larrinaga G, Perez I, Blanco L, López JL, Andrés L, Etxezarraga C, et al. Increased prolyl endopeptidase activity in human neoplasia. Regul Pept. 2010; 163: 102–106. doi: 10.1016/j.regpep.2010.03.012 PMID: 20362629

25. Larrinaga G, Blanco L, Perez I, Sanz B, Errarte P, Beitia M, et al. Prolyl endopeptidase activity is correlated with colorectal cancer prognosis. Int J Med Sci 2014; 11: 199–208. doi: 10.7150/ijms.7178 PMID: 24465166

26. Brennen WN, Isaacs JT, Denmeade SR. Rationale behind targeting fibroblast activation protein-expressing carcinoma-associated fibroblasts as a novel chemotherapeutic strategy. Mol Cancer Ther. 2012; 11: 257–266. doi: 10.1158/1535-7163.MCT-11-0340 PMID: 22323494

27. Edge SB, Byrd DR, Compton CC, Fritz AG, Greene FL, Trotti A, et al. (2010) AJCC Cancer Staging Manual, 7th Edition, Springer, New York.

28. Washington MK, Berlin J, Branton P, Bugart LJ, Carter DK, Fitzgibbons PL, et al. Protocol for the examination of specimens from patients with primary carcinoma of the colon and rectum. Arch Pathol Lab Med. 2010; 133: 1539–1551. doi:10.1043/1543-2165-133.10.1539 PMID: 19792043

29. Alponti RF, Frezzatti R, Barone JM, Alegre Vde S, Silveira PF. Dipeptidyl peptidase IV in the hypothalamus and hippocampus of monosodium glutamate obese and food-deprived rats. Metabolism 2011; 60: 234–242. doi: 10.1016/j.metabol.2009.12.031 PMID: 20153005

30. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976; 72: 248–254. PMID: 942051

31. van Hensbergen Y, Broxterman HJ, Hanemaaijer R, Jorna AS, van Lent NA, Verheul HM, et al. Soluble aminopeptidase N/CD13 in malignant and nonmalignant effusions and intraal fluid. Clin Cancer Res 2002; 8: 3747–3754. PMID: 12473585

32. Candenas L, Seda M, Noheda P, Buschmann H, Cintado CG, Martin JD, et al. Molecular diversity of voltage-gated sodium channel alpha and beta subunit mRNAs in human tissues. Eur J Pharmacol 2006; 541: 9–16. PMID: 16750188

33. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002; 3: RESEARCH0034. PMID: 12184808

34. Thirunavukarasu P, Sukumar S, Sathaiah M, Mahan M, Pragatheeshwar KD, Pingpank JF, et al. C-stage in colon cancer: implications of carcinoembryonic antigen biomarker in staging, prognosis, and management. J Natl Cancer Inst. 2011; 103: 689–697. doi: 10.1093/jnci/djr078 PMID: 21421861

35. Brennen WN, Rosen DM, Wang H, Isaacs JT, Denmeade SR. Targeting carcinoma-associated fibroblasts within the stroma with a fibroblast activation protein-activated prodrug. J Natl Cancer Inst 2012; 104: 1320–1334. doi: 10.1093/jnci/djs336 PMID: 22911669

36. Wickström M, Larsson R, Nygren P, Gulbo J. Aminopeptidase N (CD13) as a target for cancer chemotherapy. Cancer Sci. 2011; 102: 501–508. doi: 10.1111/j.1349-7006.2010.01826.x PMID: 21205077

37. Nazarian A, Lawlor K, Yi SS, Philip J, Ghosh M, Yaneva M, et al. Inhibition of circulating dipeptidyl peptidase IV activity in patients with metastatic prostate cancer. Mol Cell Proteomics 2014; 13: 3082–3096. doi:10.1074/mcp.M114.038836 PMID: 25056937

38. Cordero OJ, Imbemon M, Chiara LD, Martinez-Zorrano VS, Ayude D, De la Cadena MP, et al. Potential of soluble CD26 as a serum marker for colorectal cancer detection. World J Clin Oncol 2011; 2: 245–261. doi: 10.5306/wjco.v2.i6.245 PMID: 21773075