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Differential Expression of PP2A and Set in Hepatocellular Carcinoma: Antitumoral Effect of an Interfering Peptide Blocking the Interaction

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Abstract

Hepatocellular carcinoma is one of the most frequent cancers worldwide. Systemic treatments such as immune checkpoint or tyrosine kinase inhibitors have some efficacy but also have many adverse effects. More specifically targeted therapies are therefore needed. In this study, we investigated the anti-tumoral effect of a tumor penetrating and interfering peptide blocking the interaction between the proteins PP2A and SET. We analyzed the expression of two proteins, the phosphatase PP2A and the oncoprotein SET, in a group of samples from 21 liver cancer patients with different aggressiveness scores. Expression of PP2Aand SET was found to correlate with aggressiveness of the tumor. *In vivo* tests on xenograft models of hepatocellular carcinoma xenograft models showed an anti-tumoral effect of iRGD-IP, a tumor-penetrating and interfering peptide that blocks PP2A/SET interaction and specifically targets tumor cells suggesting that this peptide could be a strong candidate for development as therapeutic peptide for liver tumor treatment.

Keywords: Therapeutic peptide; Liver cancer; Xenograft models; PP2A; SET

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Introduction

Hepatocellular Carcinoma (HCC) is a primary liver cancer that originates from hepatocytes [1]. HCC is the sixth most frequent cancer and the fourth leading cause of cancer-related mortality worldwide. Risk factors for HCC include viral infection, alcohol abuse, non-alcoholic fatty liver disease, certain toxins, and genetic diseases. These factors cause chronic liver inflammation and fibrosis, and ultimately leading to cellular transformation and liver cancer [2,3]. Therapies against HCC include local surgical resection treatment protocols, comprising transplantation, destruction of tumors, trans-arterial chemoembolization or radioembolization and external radiotherapy. The current first-line systemic treatments use a combination of programmed cell death inhibitors (PD-1 or PD-L1) and anti-VEGF monoclonal antibodies or anti-PD-L1 and anti-CTL-4 antibodies. Second-line treatments or alternatives due to contraindications are tyrosine kinase inhibitors and/or anti-CTL-4 antibodies [2,4].

Protein Phosphatase 2A (PP2A) is a holoenzyme composed of three subunits (A, B, and catalytic) that belongs to the serine/threonine phosphatases family. PP2A regulates various cellular processes, including protein synthesis, cellular signaling, cell cycle, apoptosis, metabolism and stress responses [5]. PP2A is described as a tumor suppressor as its pharmacological inhibitor, okadaic acid, is a tumor promoter [6]. Inactivating mutations or decreased expression of PP2A subunits have been found in a broad variety of human malignancies [7]. The tumor-suppressing function of PP2A therefor makes it a potential target for novel anti-cancer therapies [6,8,9].

The SET protein is an oncoprotein that belongs to a family of multitasking proteins involved in apoptosis, transcription, nucleosome assembly and histone binding. SET is localized in the nucleus and in the cytoplasm where it plays a critical role in the regulation of normal and tumor signal transduction. High SET expression has been linked to cell growth and transformation in several

types of tumors, especially in hematological cancers [10]. Moreover, as SET inhibits PP2A by forming a complex with PP2Ac [11-13], the properties of SET are thought to be mediated by its ability to inhibit PP2A.

We previously developed a bifunctional peptide composed of a Tumor-Penetrating Peptide (TPP) that selectively internalizes into tumor hepatocytes [14-16], coupled with an Interfering Peptide (IP) that blocks the interaction between PP2A and SET [15] and induces tumor cell apoptosis [16].

Furthermore, to address IP delivery into malignant B cells, we tested four different TPPs, i.e., RPARPAR, iRGD, TT1, and Lin TT1. TPPs bind to and internalize into tumor cells where they can be used to deliver therapeutic cargos. The TPPs contain the sequence R/KXXR/K at the C terminal (CendR) and bind to Neurpilin-1 Receptor (NRP-1), which is expressed in various malignant cells. The iRGD peptide binds to the primary αβ 3/5 integrin receptor in the tumor vasculature, and after being cleaved by tumoral proteases, it binds to the NRP-1 receptor and thus internalizes into tumor cells [17,18].

Here we analyzed the expression of PP2A and SET in patient samples of HCC with different aggressivity scores and we further investigated the antitumoral effect of the tumor-penetrating and interfering peptide iRGD-IP, that blocks the interaction between PP2A/SET.

Materials and Methods

Patients and tumors

Samples of benign and tumoral liver were collected from 21 patients. All patients gave informed consent. Patient samples 7, 9 and 12 corresponded to hepatocellular adenoma, i.e., non-malignant tumors and the other patient samples corresponded to malignant primary liver tumor (Table 1). Patient 13 was a CK19 negative cholangiocarcinoma. The sample from patient 15 was not considered in the Table 1 due to a high level of necrosis. A tumor aggressiveness score was calculated based on histological or biological factors known to be associated with poor prognosis as described elsewhere. We selected six prognostic factors were taken from clinical studies, based on pathology reports after surgical resection of HCC. Each parameter was focused on tumoral cells or tissues, not on tumoral disease. We therefore excluded other prognostic factors such as patient age, or number and size of the nodules which are influenced by preoperative treatments etc. For the risk of recurrence, each parameters had a published hazard ratio of 1.5 or more, meaning that the presence of the parameter multiplied the risk of recurrence by at least x 1.5. The score was the sum of the score for the prognostic factors and it correlated with internalization of the bifunctional peptide internalization as observed in our previous study [14]. This tumor aggressiveness score included tumor encapsulation [19], tumor differentiation [20] presence of satellite nodules, microvascular invasion, macrotrabecular type [21], and log10 Alpha Fetoprotein (AFP) value [22]. Tumors that scored 0 had zero aggressiveness, tumors that scored ≤ 6 were considered moderately aggressive, and tumors that scored >6 were considered highly aggressive.

Immunohistochemistry

The immunostaining procedure was performed on formalin-fixed, deparaffinized, 3 µm-thick sections using a Ventana Benchmark Ultra platform (Roche Diagnostics, France) coupled to an UltraView visualization system (Roche Diagnostics) following the manufacturer's instructions.

The following primary antibodies were used: mouse monoclonal anti-CK19 antibody (Agilent, France) followed by CC1 antigen retrieval buffer (36 min, 95°C) and an antibody incubation time of 20 min at 20°C; mouse monoclonal anti-human hepatocyte (HepPar) (Agilent) followed by CC1 antigen retrieval buffer (64 min, 95°C) and an antibody incubation time of 32 min at 20°C; mouse monoclonal anti-human Glypican-3 (MM, France) followed by CC1 antigen retrieval buffer (64 min, 95°C) and an antibody incubation time of 32 min at 37°C; mouse monoclonal anti-human β-catenin (Roche Diagnostics) followed by CC1 antigen retrieval buffer (64 min, 95°C) and an antibody incubation time of 32 min at 37°C, and mouse monoclonal anti-human glutamine synthetase (Roche Diagnostics) followed by antigen retrieval protease (4 min, 20°C) and an antibody incubation time of 40 min at 20°C.

Protein isolation from liver tissues

A roughly 20 mg piece of liver was cut and transferred to a tube of Precellys beads CK28-R. A total of 400 µl of lysis buffer were added (Tris 50 mM pH 8, 150 mM NaCl, 1% Triton, supplemented with protease inhibitors). The tube was then placed in the Precellys Evolution machine and shaken for 30s with a pause of 30s. The process was repeated 5 times. The extracts were centrifuged after the lysis and the supernatant was transferred to a fresh tube and centrifuged at 12,000 rpm for a further 20 min at 4°C. The supernatant was recovered to estimate protein concentration, then stored at -80°C until further analysis.

Western blotting

A total of 60 µg of liver proteins were separated by SDS-PAGE, transferred to nitrocellulose and blotted with anti-PP2A or anti-SET antibodies. The membrane was then washed and incubated with PO-conjugated secondary antibody. Proteins detection was performed using the ECL system. The blot was also hybridized with anti-actin antibody to serve as an internal control. Data for quantification of the Western blots was generated by densitometry using Image J.

Xenograft model of hepatocellular carcinoma and experimental treatment

The study used 6 to 8-week-old female SCID mice were purchased from Charles River. All mice were cared for and used in conditions and protocols that were fully compliant with the governing European Council directives on the welfare of laboratory animals. All the experiments were done following the protocols and guidelines approved by the French Committee on the use of animals for scientific research.

The HepG2 cells (1×10^6 cells) in 100 µl of phosphate saline buffer were subcutaneously injected into the right flank of each mouse. Tumor progression was monitored three times a week and tumor volume were calculated using a caliper measurement-modified formula (length × (width^2/2)) and growth curves were plotted. Once the tumor volumes reached higher than 50 mm^3, treatment was initiated and the mice were randomized into two groups (7 per group): A treatment group one receiving 5 mg/kg of the peptide iRGD peptide *via* intraperitoneal injection (five days per week), and a control group (saline) as previously described [15]. Antitumoral effect was defined based on a 4 weeks-long a decrease tumor size for.

Statistical analysis

Statistical analysis was performed by between groups comparisons using ANOVA in *in vivo* data.

Results and Discussion

Clinical characterization of the patients and tumor aggressiveness classification

Samples from 21 patients were analyzed. Samples 7, 9 and 12 were hepatocellular adenomas i.e., non-malignant tumors and the rest were Hepatocellular Carcinomas (HCC) (Table 1). A tumoral and a non-tumoral tissue sample were taken from each patient. The patient population had a median age of 62 years, and was predominantly male (60%).

Clinical aggressiveness was calculated based on six parameters, i.e. Alpha-Fetoprotein (AFP), non-encapsulation, satellite nodules, microvascular invasion, differentiation and macrotrabecular type (Table 1), which were classified and scored as follows: for encapsulation, non-encapsulated = 0, partially encapsulated = 1; for differentiation, well-differentiated = 1, moderately differentiated = 2, undifferentiated = 3; for satellite nodules, positive = 1, negative = 0; for vascular invasion, positive = 1, negative = 0; for macrotrabecular type, positive = 1, negative = 0. Patient samples 7, 9 and 12 correspond to zero aggressiveness. Samples 1, 2, 4, 5, 6, 8, 10, 13, 16, 17, 19, 20 and 21 correspond to moderate aggressive HCC. Patient samples 3, 11 14 and 18 corresponded to highly aggressiveness score.

Immunohistochemical characteristics of the patients

The immunohistochemical markers that were analyzed in the patient samples were: CK19, to differentiate HCC from cholangiocarcinoma; HepPar, a marker that differentiates HCC from metastatic carcinoma [23]; GPC3, a member of the glypican family involved in progression of HCC; β-catenin, a marker of HCC development and progression [24], and glutamine synthetase [25], which may enhance metastatic potential in HCC. Absence of CK19 expression confirmed that the patient samples were HCC but not to cholangiocarcinoma. Note that control samples 7, 9 and 12 were negative for glypican expression and patient sample 13 was negative for CK19 (Table 2).

Patient samples 7, 9 and 12 corresponded to zero aggressiveness. Samples 1, 2, 4, 5, 6, 8, 10, 13, 16, 17, 19, 20 and 21 corresponded to moderately-aggressive HCC. Patient samples 3, 11, 14 and 18 corresponded to highly-aggressive HCC. However, some of the immunohistological markers failed to correlate with and PP2A or SET expression.

Differential expression of PP2Ac and SET proteins in hepatocellular carcinoma

We have analyzed PP2Ac expression in a total of 21 patient liver samples showing different degrees of aggressiveness (Table 1). Patient samples 7, 9 and 12 were non-tumoral samples. Control sample 12 showed a similar expression of PP2Ac between non-tumoral and tumoral tissue whereas control samples 7 and 9 showed higher level of PP2Ac expression in non-tumoral than in tumoral tissue (Figure 1). The rest of the patients were divided into three groups, according to PP2A expression level: One group in which PP2Ac expression was is higher in tumoral tissue than in non-tumoral tissue (patients samples 1, 3, 4, 8, 18, 19 and 20), a second group in which PP2Ac expression was higher in non-tumoral than in tumoral tissue (patients samples 5, 10, 1, 12 and 21) and a third group in which PP2Ac expression was similar between tumoral and non-tumoral tissue (patients samples 6, 13, 14, 16 and 17) (Figure 1). Actin expression was used as an internal control of protein loading. We also calculated the ratio of PP2Ac expression to actin expression (Figure 1).

We also analyzed the SET expression level of SET in the same

Table 1: Clinical characteristics of the patients.

N°	Sex	Age	Tumor	AFP (ng/ml)	AFPlog10	Partial capsule	Satellite nodule	Microvasc. Invasion	Differentiation	Macrotrabecular	Aggressiveness	Class
1	M	73.9	HCC	8.6	0	1	0	0	2	1	4	medium
2	M	68.5	HCC	3.7	0	0	0	0	2	0	2	medium
3	F	83	HCC	1298	3	0	1	0	2	1	7	high
4	M	51.8	HCC	20.1	1	0	0	0	1	0	2	medium
5	F	72.1	HCC	10.4	1	0	0	1	2	0	4	medium
6	M	68	HCC	12.5	1	1	1	0	2	0	5	medium
7	F	39.7	noHCC	1.1	0	0	0	0	0	0	0	null
8	F	59.1	HCC	30.6	1	0	0	0	1	0	2	medium
9	F	45.5	noHCC	4.8	0	0	0	0	0	0	0	null
10	M	68.4	HCC	29	1	1	0	0	2	0	4	medium
11	F	35.3	HCC	385.9	2	0	0	1	2	1	6	high
12	F	46.7	noHCC	5.3	0	0	0	0	0	0	0	null
13	M	45.9	CCK	4.1	0	0	0	0	2	0	2	medium
14	M	41.2	HCC	340839	5	0	0	1	3	0	9	high
16	F	90.1	HCC	18.6	1	1	0	0	2	0	4	medium
17	M	65.3	HCC	8.7	0	1	0	0	3	0	4	medium
18	M	74.4	HCC	42.4	1	1	1	0	3	0	6	high
19	M	61.6	HCC	2.1	0	0	1	0	2	1	4	medium
20	M	86.7	HCC	6.1	0	0	0	0	1	0	1	medium
21	M	67.8	HCC	2.59	0	0	0	0	2	0	2	medium

CCK: Cholangiocarcinoma; HCC: Hepatocellular Carcinoma

* Number 15: Necrotic tissue not retained.

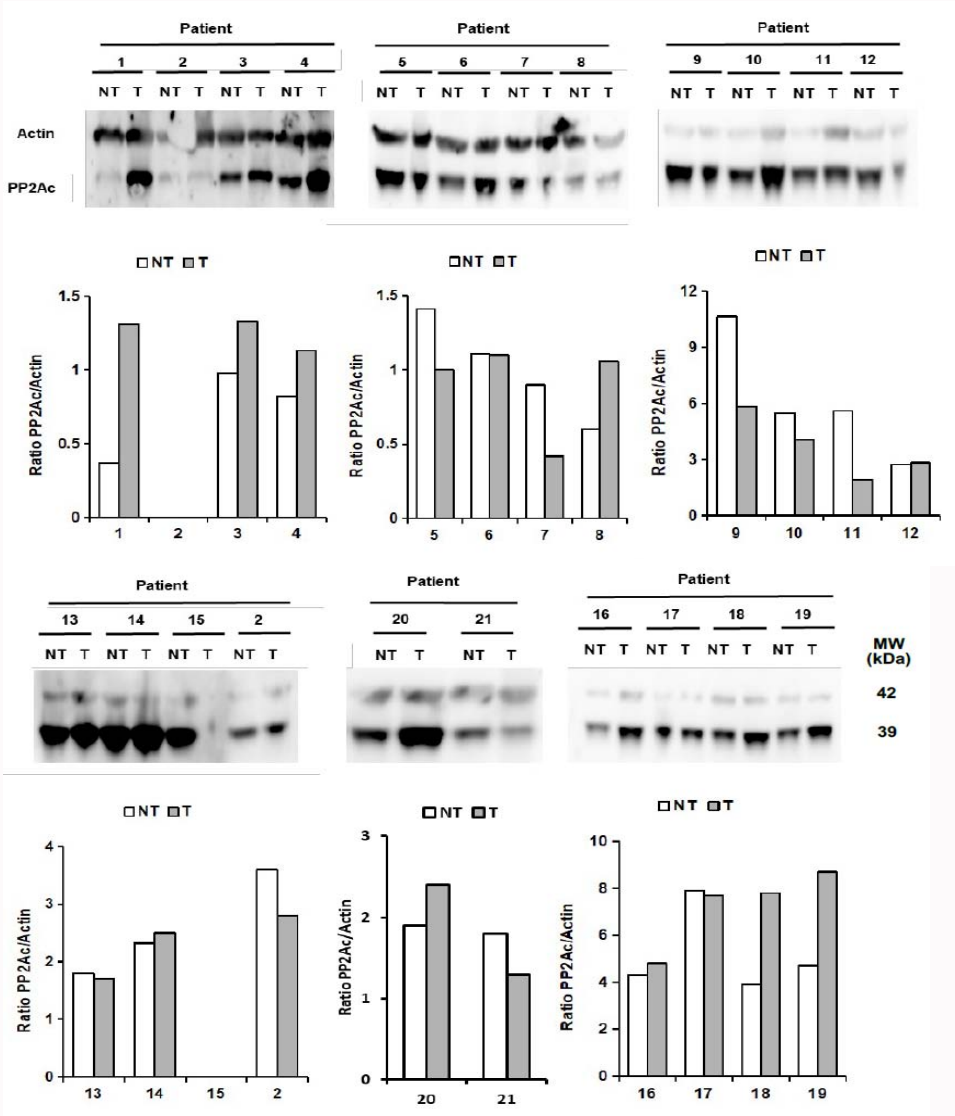

Figure 1: Western blot analysis of PP2A expression in 21 patient samples for of Tumoral (T) and Non-Tumoral (NT) liver tissue. The molecular weight of the proteins is shown. Actin expression was used as an internal control of protein loading. The figure shows the PP2Ac/Actin expression ratio.

group of samples. Like for PP2Ac, the patients were again divided into three groups: One group in which SET expression was higher in tumoral tissue that in non-tumoral tissue (samples 2, 3, 5, 6, 13, 14, 17, 18, 19 and 20), a second group in which SET expression was higher in non-tumoral tissue than in tumoral tissue (samples 1, 4, 9, 10, 12 and 21) and a third group in which SET expression was similar between tumoral and non-tumoral tissue (samples 7, 8 and 11) (Figure 2). The figure shows SET expression to actin expression ratio.

Correlation between PP2A/SET expression and aggressiveness score

To go further, we analyzed whether the patient samples with a high level of PP2A expression also showed a high level of SET expression, and whether there was a relationship between a high PP2A and SET expression levels and aggressiveness score. As illustrated in Figure 3, the patient samples that expressed a high level of PP2A and SET (samples 3, 14 and 18) are also the samples with the highest aggressiveness score. Patient sample 11 express only high level of SET and shows high aggressiveness score. The patients' samples on the quadrant labelled" B" in Figure 3, showed high expression of both proteins show and an average of aggressiveness score of 5.4 which was significantly higher, than the mean for the rest of the sample population (aggressiveness score 2.9).

Antitumoral effect of iRGD-IP on liver xenograft models

Given that iRGD-IP peptide penetrates into tumoral primary hepatocytes and that it is very stable against degradation by serum proteases, we decided to evaluate its antitumoral effect in a mouse xenograft model of liver cancer generated using the HepG2 liver cell line. The mice were treated with the peptide at a dose of 5 mg/kg for 5 days per week. Controls were treated with saline solution (control group). As shown in Figure 4A, the peptide treatment had an antitumoral effect in the liver xenograft model, reflected by a roughly 46% decrease in the tumor size, compared to non-treated controls.

To assess the potential toxicity of the iRGD-IP peptide, we compared the body weight dynamics of the mice in the treated group *vs.* the control group. As shown in Figure 4B, there was no significant reduction in body weight in the treated mice compared to controls,

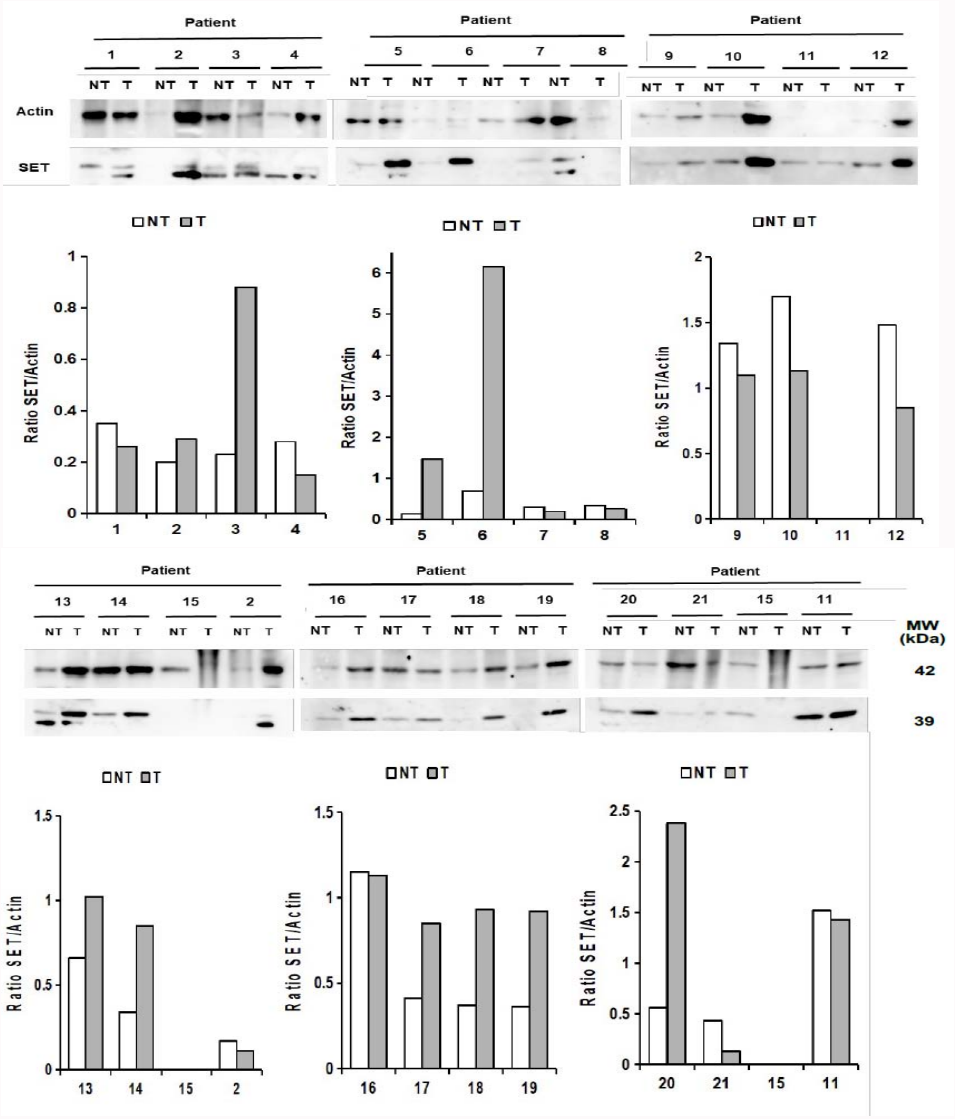

Figure 2: Western blot analysis of SET expression in 21 patient samples of Tumoral (T) and Non-Tumoral (NT) liver tissue. Actin expression was used as an internal control of protein loading. The figure shows the SET/Actin expression ratio and molecular weight of the proteins.

which suggests that the peptide has not toxicity.

Liver cancer is growing in prevalence, and is projected to affect up to one Million of people in the next few years [2,26]. The most common form of liver cancer is Hepatocellular Carcinoma (HCC) which accounts for approximately 90% of the of liver cancer cases with around 25% of which are undruggable [3,27]. The definition of liver cancer has been modified in order to use personalized therapies for treatment [1]. The pathogenesis of HCC is a complex process and the classification of HCC has been modified on order to use personalized therapies for treatment [28-33].

There have been reports of therapeutic approaches for specifically targeting tumoral HCC cells. Interfering peptides that block intracellular protein-protein interactions are emerging as promising agents [34,35]. The serine-threonine phosphatase PP2A commonly shows altered expression or activation in cancer [8,10,36]. The physiological inhibitor of PP2A is the oncoprotein SET, which associates with the catalytic subunit of PP2A and blocks its activation. We previously generated several tumor-penetrating and interfering peptides blocking the PP2A/SET interaction that specifically penetrate into tumoral hepatocytes and induce apoptosis [15]. Here we tested the antitumoral effect of one of these peptides in a xenograft models of liver cancer.

Various clinical parameters have been used to define an HCC aggressiveness score including tumor size, multifocality, presence of portal vein thrombus and alpha-fetoprotein levels [37,38]. Here we defined an aggressiveness score based on the parameters indicated in Table 1.

According to these criteria, we classified the patients into three groups: non aggressive (non- malignant tumors), moderately aggressive tumor (score up to 5) and highly aggressive tumor (score of 6 and higher). The results showed a relationship between the aggressiveness score and the level of PP2Ac and SET expression (patients 3, 14 and 18).

Treatment of a xenograft mouse model of liver cancer with the peptide iRGD-IP lead to a substantial reduction in tumor size, which makes iRGD-IP a good candidate for clinical development

Table 2: Immunohistochemical characteristics of the patients.

N°	Tumor	HepPar-1	Glypican-3	CK19	Glutamine synthetase	β-catenin
1	HCC	+++	-	-	+	+
2	HCC	+++	+	-	+	-
3	HCC	+++	+	-	-	-
4	HCC	++	+++	-	-	-
5	HCC	+++	+++	-	+	-
6	HCC	+++	-	-	-	-
7	adenoma	+++	-	NA	-	-
8	HCC	+++	+++	-	+	-
9	adenoma	+++	-	NA	-	-
10	HCC	+	+	-	-	-
11	HCC	+++	+	-	-	-
12	adenoma	+++	-	NA	+	-
13	CCK	-	-	-	NA	-
14	HCC	+	+	+	-	-
16	HCC	+++	+++	-	-	-
17	HCC	-	+	+	-	-
18	HCC	+	++	-	++	-
19	HCC	+++	++	-	-	-
20	HCC	+++	-	-	++	-
21	HCC	+++	-	-	+	-

CCK: Cholangiocarcinoma; HCC: Hepatocellular Carcinoma; NA: Not Applicable

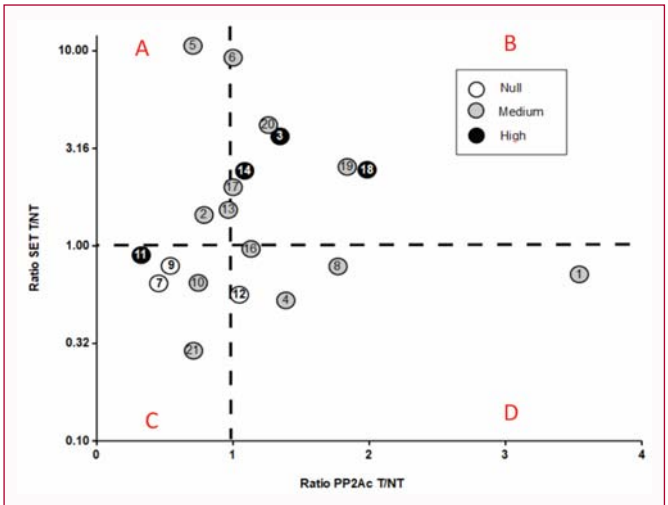

Figure 3: Patient distribution plot.
Synthetic view of PP2A and SET expression and aggressiveness score plotted for each patient White circles, null aggressiveness; grey circles, medium aggressiveness; black circles, high aggressiveness. p 0.05 for the aggressiveness score of quadrant B versus A, C and D.

as a therapeutic peptide against HCC. We cannot exclude that the antitumoral effect may vary depending on whether the response to iRGD-IP peptide is analyzed using a subcutaneous xenograft model or an orthotopic xenograft model. The involvement of several tumor-dependent steps makes the mechanism of action of TPPs highly selective. First, the bi-functional peptide iRGD-IP binds to its primary receptor, the integrin. Then, after proteolytic cleavage by tumoral proteases to expose the CendR motif, its binds to the NRP-1 receptor. There are several lines of evidence to suggest a correlation between the level of NRP-1 expression and tumor aggressiveness [39,40]. We have previously shown a correlation between integrin receptor expression, internalization of the peptide and tumor aggressiveness [14].

Uncontrolled tumor cell proliferation and escape from apoptosis plays an important role in HCC. Patients in late-stage HCC are normally treated with chemotherapy [41]. However, the prognosis of these patients is compromised due to the toxic side effects of the drug and the emergence of drug-resistant tumors [42]. As a result, there is a real need to search for new and more targeted therapies for liver cancer.

Several PP2A protein inhibitors have been described to date. One of these is SET, which contributes to tumorigenesis by forming a complex with PP2A [43]. SET expression levels have been associated with more aggressive disease in ovarian cancer and chronic lymphocytic leukemia [43,44]. Several efforts to restore PP2A activity have focused on interfering with the PP2A/SET interaction [45,46] which emerges as a promising protein/protein interaction to be modulated as a therapeutic target.

The peptide OP449 (COG 112), composed of a cell penetrating peptide associated to the residues 133-149 of the apolipoprotein E was described as a SET-inhibiting ApoE mimetic that blocks PP2A/SET interaction [43,45]. Here we present a similar strategy with some differences. First, we used a tumor-targeted peptide, the tumor-penetrating peptide iRGD, which is highly stable against proteases degradation and can thus serve for *in vivo* applications.

Most importantly, the interfering peptide has been specifically designed to target the interaction between PP2A and SET. This direct strategy specifically targeting PP2A/SET interaction means that we do not affect the other interactions of PP2A or SET, thus avoiding side effects. Another reported inhibitor of SET is FTY720, which binds to SET in the region responsible for histidine chaperone activity [47].

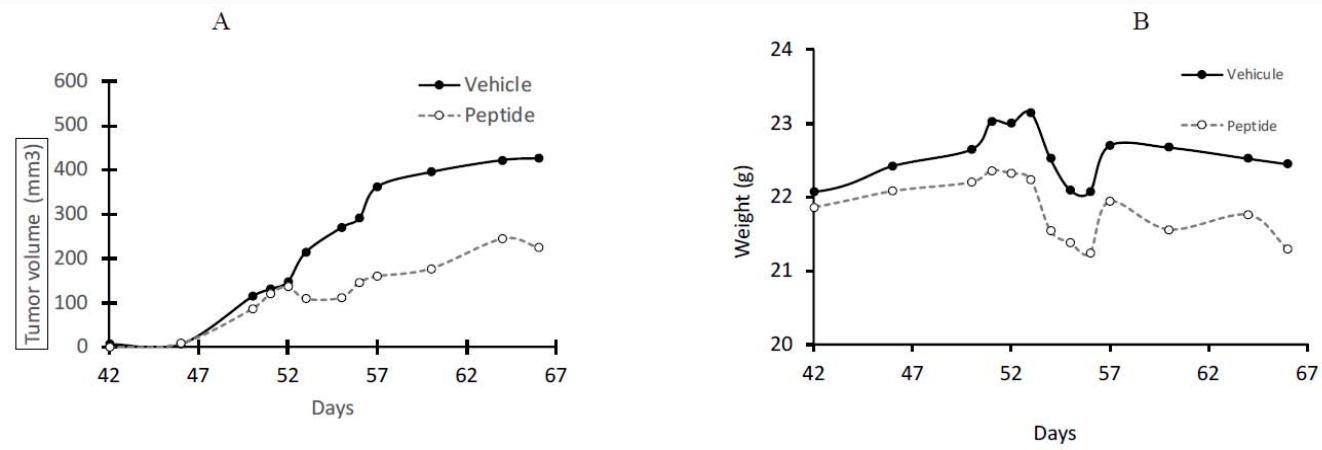

Figure 4: The iRGD-IP peptide has antitumoral activity in the xenograft mouse model of liver cancer. A) Mice were IP injected with 5 mg/kg of the peptide on 5 days per week for 4 weeks. Tumor size was monitored over time. Solid black line: control group. Dashed grey line: treatment group. Statistical comparisons were performed using ANOVA. B) Percent of the initial body weight in the xenograft mouse model of liver cancer treated with iRGD-IP peptide or with saline solution (control).

The phosphatase PP2A and its physiological inhibitor, the oncoprotein SET, are involved in HCC and other types of cancers such as hematological cancers, where they negatively regulate a number of signaling pathways such as including Wnt-β-catenin, PI3K, MAPK and more [48,49]. PP2A inhibitors have shown proven a therapeutic effect against HCC in clinical trials [50-52], which points to PP2A as a promising target for HCC treatment. However, the constitutive expression of PP2A in all the cells together with the array of partners and signaling pathways controlled by this phosphatase have posed a barrier to the use of PP2A as a target for the development of therapeutic agents. Moreover, in clinical trials, phosphatase inhibitors have shown a toxic effect against non-tumoral liver tissues [8,10,36], indicating that the therapeutic efficacy of these treatments hinges on a precise cancer-targeted delivery system. We have addressed this challenge by generating bi-functional peptides composed of a Tumor Penetrating Peptide (TPP) coupled to an Interfering Peptide (IP) blocking the interaction between the phosphatase PP2A and SET. Our bi-functional peptide does not affect other interactions of PP2A or SET with their partners, and only has an effect on the pool of PP2A associated to SET. This peptide has shown proven anti-tumoral effect on xenograft models of liver cancer without side effects, which makes it a strong candidate for development as a therapeutic peptide against HCC.

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