

Combined cetuximab and trastuzumab are superior to gemcitabine in the treatment of human pancreatic carcinoma xenografts

C. Larbouret^{1,2,3,4}, B. Robert^{1,2,3,4}, C. Bascoul-Mollevis⁴, F. Penault-Llorca⁵, A. Ho-Pun-Cheung⁴, S. Morisseau^{1,2,3,4}, I. Navarro-Teulon^{1,2,3,4}, J.-P. Mach⁶, A. Pèlegri^{1,2,3,4*} & D. Azria^{1,2,3,4,7}

¹Institut de Recherche en Cancérologie de Montpellier, Montpellier; ²Institut National de la Santé et de la Recherche Médicale, U896, Montpellier; ³Université Montpellier1, Montpellier; ⁴CRLC Val d'Aurelle Paul Lamarque, Montpellier; ⁵Department of Pathology Centre Jean Perrin, Clermont-Ferrand; ⁶Institut de Biochimie, Université de Lausanne, Epalinges, Switzerland and ⁷Department of Radiotherapy CRLC Val d'Aurelle Paul Lamarque, Montpellier, France

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Background: Pancreatic carcinoma remains a treatment-refractory cancer with a poor prognosis. Here, we compared anti-epidermal growth factor receptor (EGFR) and anti-HER2 monoclonal antibodies (2mAbs) injections with standard gemcitabine treatment on human pancreatic carcinoma xenografts.

Materials and methods: Nude mice, bearing human pancreatic carcinoma xenografts, were treated with either combined anti-EGFR (cetuximab) and anti-HER2 (trastuzumab) or gemcitabine, and tumor growth was observed.

Results and conclusion: In first-line therapy, mice survival was significantly longer in the 2mAbs group compared with gemcitabine ($P < 0.0001$ for BxPC-3, $P = 0.0679$ for MiaPaCa-2 and $P = 0.0019$ for Capan-1) and with controls ($P < 0.0001$). In second-line therapy, tumor regressions were observed after replacing gemcitabine by 2mAbs treatment, resulting in significantly longer animal survival compared with mice receiving continuous gemcitabine injections ($P = 0.008$ for BxPC-3, $P = 0.05$ for MiaPaCa-2 and $P < 0.001$ for Capan-1). Therapeutic benefit of 2mAbs was observed despite K-Ras mutation. Interestingly, concerning the mechanism of action, coinjection of F(ab')₂ fragments from 2mAbs induced significant tumor growth inhibition, compared with controls ($P = 0.001$), indicating that the 2mAbs had an Fc fragment-independent direct action on tumor cells. This preclinical study demonstrated a significant improvement of survival and tumor regression in mice treated with anti-EGFR/anti-HER2 2mAbs in first- and second-line treatments, compared with gemcitabine, independently of the K-Ras status.

Key words: EGFR, gemcitabine, HER2, monoclonal antibodies, pancreatic carcinoma

Introduction

Pancreatic cancer is the fourth leading cause of cancer death in both men and women. Currently, most pancreatic cancer patients die within a year of diagnosis. When the tumor becomes symptomatic, 60%–80% of the patients already have locally advanced or metastatic disease, allowing essentially palliative therapy, with a 5-year survival rate of <5% [1].

Recent evaluation of gemcitabine-based combination chemotherapy trials in advanced pancreatic cancer has demonstrated significant, but low response rates and disappointing effects on survival [2]. The strategies of earlier attack on cancer by perioperative adjuvant or neo-adjuvant therapy were expected to be more promising. However, the

recently reported retrospective and prospective analyses of adjuvant chemoradiotherapy of pancreas carcinoma [3], as well as the results of preoperative chemoradiation therapies [4], have again demonstrated significant, but very modest therapeutic gains. Similarly, the results from a recent phase III trial, evaluating the advantage of the association of erlotinib with gemcitabine, showed a significant, but low improvement, as compared with gemcitabine-alone treatment, with a median survival of 6.24 versus 5.91 months, as well as a 1-year survival of 23% versus 17%, respectively [5].

In the field of mAb therapies and following the demonstration of the expression of both epidermal growth factor receptor (EGFR) and HER2 in pancreatic carcinomas [6, 7] and the known implication of those receptors in the malignant phenotype [8–11], we have recently demonstrated that the coinjection of anti-EGFR and anti-HER2 mAbs had a significant synergic effect in the treatment of human pancreatic carcinoma xenografts, as compared with the effect of each mAb alone [12]. Our results were recently confirmed and

*Correspondence to: Dr A. Pèlegri, Institut de Recherche en Cancérologie de Montpellier, CRLC Val d'Aurelle Paul Lamarque, 34298 Montpellier Cedex 5, France. Tel: +33-0-467613032; Fax: +33-0-467613787; E-mail: Andre.Pelegri@valdorel.fnclcc.fr

extended by the demonstration that different pairs of anti-HER2 mAbs had a synergistic antitumor effect [13].

Here, we are evaluating, if the combined anti-EGFR/anti-HER2 mAbs (2mAbs) targeted therapy can result in better efficacy than the current standard chemotherapy, gemcitabine, in a first- and second-line therapy.

methods

monoclonal antibodies and drugs

Cetuximab was purchased from Merck KGaA (Darmstadt, Germany), trastuzumab from Roche Pharma AG (Grenzach-Wyhlen, Germany) and gemcitabine from Lilly France (Pages, France). F(ab')₂ fragments of cetuximab and trastuzumab were prepared by pepsine digestion followed by filtration on a Superdex 200 column. Absence of intact mAb contamination was checked by lack of any cytotoxicity induction by natural killer cells on antigen-positive target cells [14].

cell lines

BxPC-3 and MiaPaCa-2 pancreatic cell lines were from ATCC (Rockville, MD). The Capan-1 was provided by L. Buscail (Toulouse, France).

xenograft study and treatment procedure

All *in vivo* experiments were carried out in compliance with the national regulations and ethical guidelines for experimental animal studies in an accredited establishment (Agreement No. B34-172-27). Six-week-old female athymic mice (Harlan, Le Malcourlet, France) were xenografted subcutaneously with BxPC-3 (3.5×10^6), MiaPaCa-2 (5×10^6) and Capan-1 (10×10^6) cells. Tumor-bearing mice were randomized when tumors reached a minimum of 50 mm³ and killed when tumor reached a volume larger than 1000 mm³.

In first line, mice were treated twice a week either by intraperitoneal injections of combined trastuzumab/cetuximab (ratio 1 : 1; 2 mg/kg of each mAb) or gemcitabine (150 mg/kg) diluted in 0.15-ml saline.

In second line, 20 mice were treated twice a week with gemcitabine alone (150 mg/kg). For 10 mice presenting a tumor progression (volume increase at least two-fold from initial measurement) the gemcitabine treatment was replaced by the combined trastuzumab/cetuximab intraperitoneal injections twice a week (ratio 1 : 1; 2 mg/kg). The other 10 mice were continuously treated by gemcitabine.

To determine the implication of the Fc fragment portion of antibodies, BxPC-3 xenografted mice were treated twice a week for 4 weeks with F(ab')₂ fragments from both trastuzumab and cetuximab (ratio 1 : 1; 1.35 mg/kg of each fragment) or cetuximab F(ab')₂ alone, at the same dose, or intact trastuzumab and cetuximab (ratio 1 : 1; 2 mg/kg). The concentration of fragments was adjusted to be at the same molarity (2 μM) as the intact antibodies.

Tumor dimensions were measured with a caliper and the volumes calculated by the formula: $D_1 \times D_2 \times D_3/2$. In Figure 3, tumor growth is expressed as the log of tumor progression: $\log [\text{final volume } (t)/\text{initial volume } (t_0)]$.

K-Ras mutation analysis

High-molecular weight DNA was extracted using a Qiamp DNA mini kit (Qiagen, Courtaboeuf, France). Direct sequencing of the K-Ras gene codon 12 and 13 was done with a 3130 Genetic Analyzer (Applied Biosystems, Courtaboeuf, France), using the Bigdye terminators v1.1 cycle sequencing kit (Applied Biosystems).

immunohistochemical analyses

Seven days after treatment, tumors were harvested and fixed 12 h in buffered formalin and embedded in paraffin.

Ki-67 (MiB1; Dako Corporation, Glustrup, Denmark), EGFR (3C6; Ventana Medical Systems, Illkirch, France) and pEGFR (tyr 1068, 1H12; Cell Signalling Technology, Beverly, MA) immunostaining were carried out on 3-mm sections with a BenchmarkXT immunostainer (Ventana Medical Systems). Sections were scored under light microscopy by two independent pathologists, who analyzed five different fields per section. Error bars correspond to results obtained in the different fields.

For EGFR the two plus or more intensity staining of tumor cells membrane was scored positively, while for Ki-67 index and pEGFR the percentage of tumor cells with one plus nuclear and/or cytoplasmic staining was recorded positively. The results are expressed on histograms.

western blot analysis

Seven days after treatment, tumors were harvested and lysed as described [12].

After electrophoresis, the proteins were transferred to a polyvinylidene difluoride membranes (Millipore Co., Bedford, MA) which were saturated in phosphate-buffered saline containing 0.1% Tween 20 and 5% nonfat dry milk and then incubated with the antibodies against the phosphorylated forms of AKT (Cell Signaling Technology). To ensure equal loading, immunoblots were also probed with anti-glyceraldehyde-3-phosphate dehydrogenase antibody (Chemicon International, Millipore SAS, Molsheim, Australia).

statistical analyses

A linear mixed regression model was used to determine the relationship between tumor growth and the number of days after implantation. The fixed part of the model included variables corresponding to the number of days after implantation and to different groups. Interaction terms were built into the model. Random intercept and random slope were included to take into account time effect. The coefficients of the model were estimated by maximum likelihood and considered significant at the 0.05 level.

Survival rates were estimated from the date of the xenograft until the date when the tumor reach a volume of 1000 mm³ using the Kaplan–Meier method. Median survival was presented with 95% confidence intervals. Survival curves were compared using the log-rank test. Statistical analysis was carried out using STATA 10.0 software.

results

pancreatic cell lines characteristics

Immunohistochemical analyses of the three human pancreatic carcinoma xenografted in nude mice showed a very low HER2 expression, but a high level of EGFR, classified as +++ for both BxPC-3 and Capan-1 and as ++ for MiaPaCa-2 (Table 1). When tested by flow cytometry [15], the three pancreatic carcinoma cell lines showed a moderate expression of HER2 (Table 1).

Table 1. EGFR and HER2 expression and K-Ras status of the three human pancreatic cell lines

Cell lines	EGFR		HER2		Sequencing
	IHC	FACScan (MFI)	IHC	FACScan (MFI)	K-Ras status
BxPC-3	3+	156	ND	37.0	WT
MiaPaCa-2	2+	31	ND	10.7	M
Capan-1	3+	135	ND	10.4	M

EGFR, epidermal growth factor receptor; IHC, immunohistochemistry; ND, nondetectable; MFI, mean fluorescence intensity; WT, wild type; M, mutation.

The presence of G12C mutation was confirmed in the MiaPaCa-2 and Capan-1 carcinoma cell line, while it was not observed in BxPC-3 line (Table 1) [16, 17].

first-line combined antibody therapy versus gemcitabine

Gemcitabine dose was fixed at 150 mg/kg per injection and antibody doses were selected on the basis of our previous experiments [12].

In the three human pancreatic carcinoma xenografts, the survival of mice treated with the 2mAbs was significantly greater compared with gemcitabine ($P = 0.0006$ for BxPC-3, $P = 0.0679$ for MiaPaCa-2 and $P = 0.0018$ for Capan-1) and control groups ($P = 0.0001$ for BxPC-3, $P = 0.0006$ for MiaPaCa-2 and $P = 0.0025$ for Capan-1) (Figure 1). Median delays for each of the three tumors to reach a volume of 1000 mm^3 under 2mAbs, gemcitabine or no treatment are reported in Table 2. Only the groups treated with the 2mAbs showed complete responses with 20%–33% of tumor-free mice after 2 months of follow-up (Table 2).

second-line combined antibody therapy after progression under gemcitabine

The two mAbs were injected to mice with larger tumors, compared with first-line therapy, since a minimum two-fold tumor progression had to be recorded at the time of 2mAbs treatment initiation (Figure 2). In BxPC-3 and Capan-1 models significant tumor regressions were observed when the mice received 2mAbs, compared with continuous gemcitabine injection group ($P < 0.001$), while in the MiaPaCa-2 model, 3 of 10 mice showed a marked tumor regression and the others showed only a stabilization of tumor growth ($P = 0.003$). Despite moderate tumor regrowth, observed after tumor regressions or stabilization, survival was significantly longer in mice from the three pancreatic carcinoma models, treated in second line with 2mAbs compared with mice treated only with gemcitabine ($P = 0.008$ for BxPC-3, $P = 0.052$ for MiaPaCa-2 and $P = 0.0018$ for Capan-1).

immunohistochemistry and western blot analysis

To assess the *in vivo* mechanisms underlying the antitumor activity of the two mAbs combination, we analyzed in BxPC-3 tumor xenografts, by immunohistochemistry (IHC), the EGFR expression, the EGFR phosphorylation level and the proliferative Ki-67 index, after 1 week of 2mAbs or gemcitabine treatment. As shown in Figure 3, the 2mAbs treatment induced a marked decrease of the EGFR expression, EGFR phosphorylation and Ki-67 index compared with untreated controls and gemcitabine treatment. The AKT phosphorylation after the same two treatments was assessed by western blot analysis, which showed almost complete inhibition of P-AKT after 2mAbs treatment and only minor inhibition after gemcitabine treatment compared with untreated tumor control.

comparison of $F(ab')_2$ fragments from the 2mAbs with intact antibodies

In order to separately analyze the *in vivo* direct effect of the mAbs binding sites on the EGFR and HER2 receptor, as

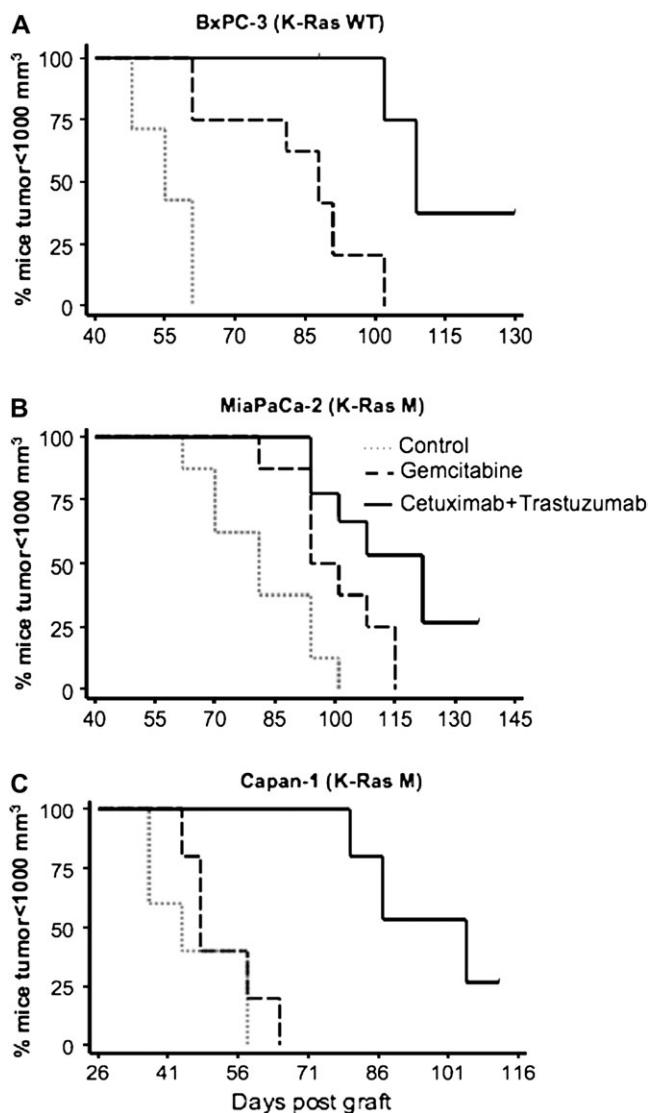


Figure 1. Kaplan–Meier survival curves of first-line combined anti-epidermal growth factor receptor and anti-HER2 monoclonal antibodies versus gemcitabine therapy. Percentage of mice with a tumor volume $<1000 \text{ mm}^3$ as a function of time after graft under either combined anti-epidermal growth factor receptor and anti-HER2 mAbs or gemcitabine or no treatment controls. K-Ras WT, K-Ras wild type; K-Ras M, K-Ras mutation.

compared with the Fc fragment-dependent effector functions of the antibodies, $F(ab')_2$ fragments from both cetuximab and trastuzumab were prepared and their therapeutic properties evaluated. As shown in Figure 4, the $F(ab')_2$ fragments from the two mAbs had a significant antitumor effect compared with untreated controls ($P < 0.001$), as well as with $F(ab')_2$ from anti-EGFR mAb alone, which had no antitumor effect ($P = 0.510$). As expected, injection of the two intact mAbs had a more pronounced antitumor effect compared with the two fragments ($P = 0.002$).

discussion

This preclinical study demonstrates a significant improvement of survival and tumor regression in mice treated with combined

Table 2. Median survival and percent of cured mice after first-line treatment

Xenograft	Treatment	Median ^a survival	Benefit ^b	Tumor-free mice % (number)
BxPC-3	Control	55		0
	Gemcitabine	88	+33	0
	2mAbs	109	+54	33 (3/9)
MiaPaCa-2	Control	81		0
	Gemcitabine	94	+13	0
	2mAbs	122	+41	22 (2/9)
Capan-1	Control	44		0
	Gemcitabine	48	+4	0
	2mAbs	105	+61	20 (2/10)

^aMedian: days after graft where 50% of the mice present a 1000 mm³ tumor volume.

^bBenefit: gain in days of the treatment versus control group.
2mAbs, anti-epidermal growth factor receptor and anti-HER2 monoclonal antibodies

anti-EGFR (cetuximab) and anti-HER2 (trastuzumab) mAbs in first and second line of treatment compared with gemcitabine, the gold standard in pancreatic cancers [2].

Of particular interest was the positive impact of this treatment despite the low HER2 expression by all three target carcinomas and despite the presence of a K-Ras mutation on two of them. It was recently shown that the efficacy of cetuximab in metastatic colorectal carcinoma was almost abolished in K-Ras-mutated tumors [18]. In pancreatic cancer, where K-Ras mutations are the norm (90%), trials evaluating cetuximab plus gemcitabine–cisplatin versus gemcitabine–cisplatin did not increase response or survival of patients [19]. Here, a clear therapeutic benefit of cetuximab and trastuzumab combination was observed in two K-Ras-mutated pancreatic carcinomas, suggesting that the lack of clinical benefit of anti-EGFR mAb could be bypassed by combining this mAb with the anti-HER2 mAb.

All three tested pancreatic carcinoma lines responded better to 2mAbs whatever their respective sensitivity to gemcitabine. In case of high sensitivity to chemotherapy (BxPC-3), i.e. in the best condition of gemcitabine efficacy, 2mAbs significantly increased tumor responses and cured three of nine mice, while no cure was obtained with gemcitabine.

The mechanism of action of the synergism of the two anti-EGFR and HER2 mAbs is not yet entirely understood, as is the case for several approved mAbs whose mode of action is not entirely unraveled. It was shown that an anti-EGFR mAb could increase the *in vitro* growth inhibition of an anti-HER2 mAb [9] or that two anti-EGFR and HER2 mAbs had a synergistic effect in the down-regulation of the receptor tyrosine kinases activity [10]. However, our *in vivo* results were the first to demonstrate unequivocally that the two anti-EGFR and HER2 mAbs can act synergistically in the treatment of human carcinoma xenografts [12]. Thus, we concentrated our effort on some *in vivo* experiments that could shed some light on the mechanism of action of 2mAbs.

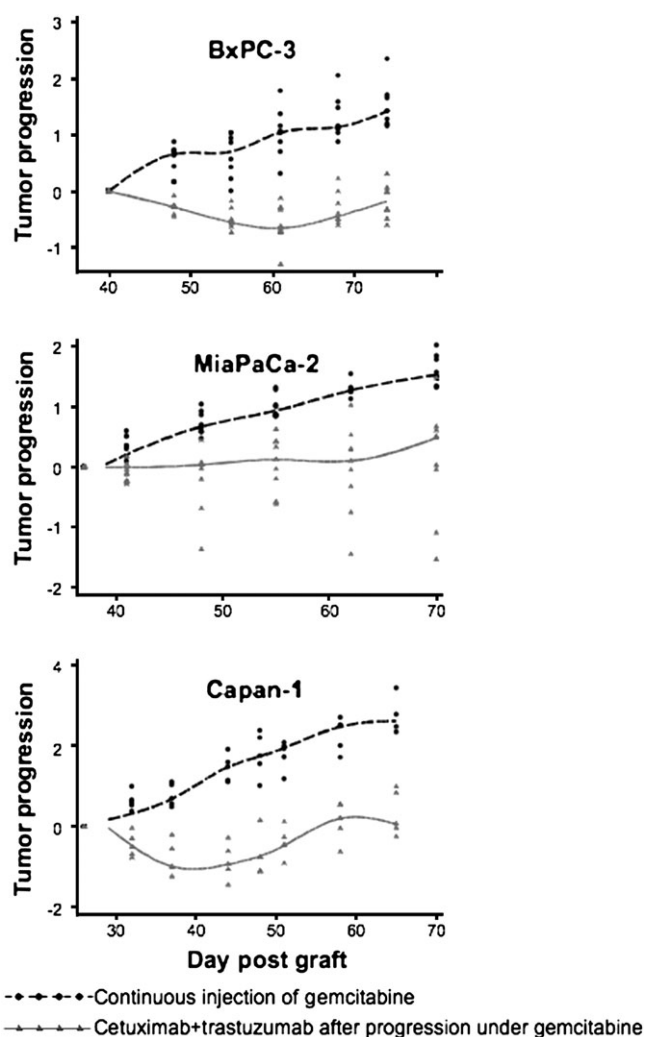


Figure 2. Tumor size evolution of second-line combined anti-epidermal growth factor receptor and anti-HER2 monoclonal antibodies (2mAbs) therapy after gemcitabine progression. Pancreatic carcinoma-bearing mice were first treated with gemcitabine. At day 26, 37 or 40 for Capan-1, MiaPaCa-2 and BxPC-3, respectively, half of the mice in each tumor group were treated with the 2mAbs combination, while the other half received continuous gemcitabine treatment.

First, we showed that the 2mAbs induced a decrease of the proliferation index Ki-67 and a down-regulation of the EGFR. These results were in agreement with those from other groups obtained after the injection of a single anti-EGFR or anti-HER2 mAbs [9–11] or by treatment with two mAbs directed against different epitopes of either EGFR [20] or HER2 receptor [10].

Second, our more original *in vivo* results were obtained by the combined injection of F(ab')₂ fragments from cetuximab and trastuzumab in order to determine whether the tumor growth inhibition observed with the two intact mAbs was due to a direct action of the two antibody-binding sites on the two receptors, by inhibiting their dimerizations or triggering of a negative intracellular signal, as indicated by *in vitro* results [8–11] or if it was due to an antibody-dependent cellular cytotoxicity enhancement, as indicated by *in vivo* experiments [21]. Our results show for the first time *in vivo* that F(ab')₂ from two anti-EGFR and HER2 receptor mAbs significantly inhibited the

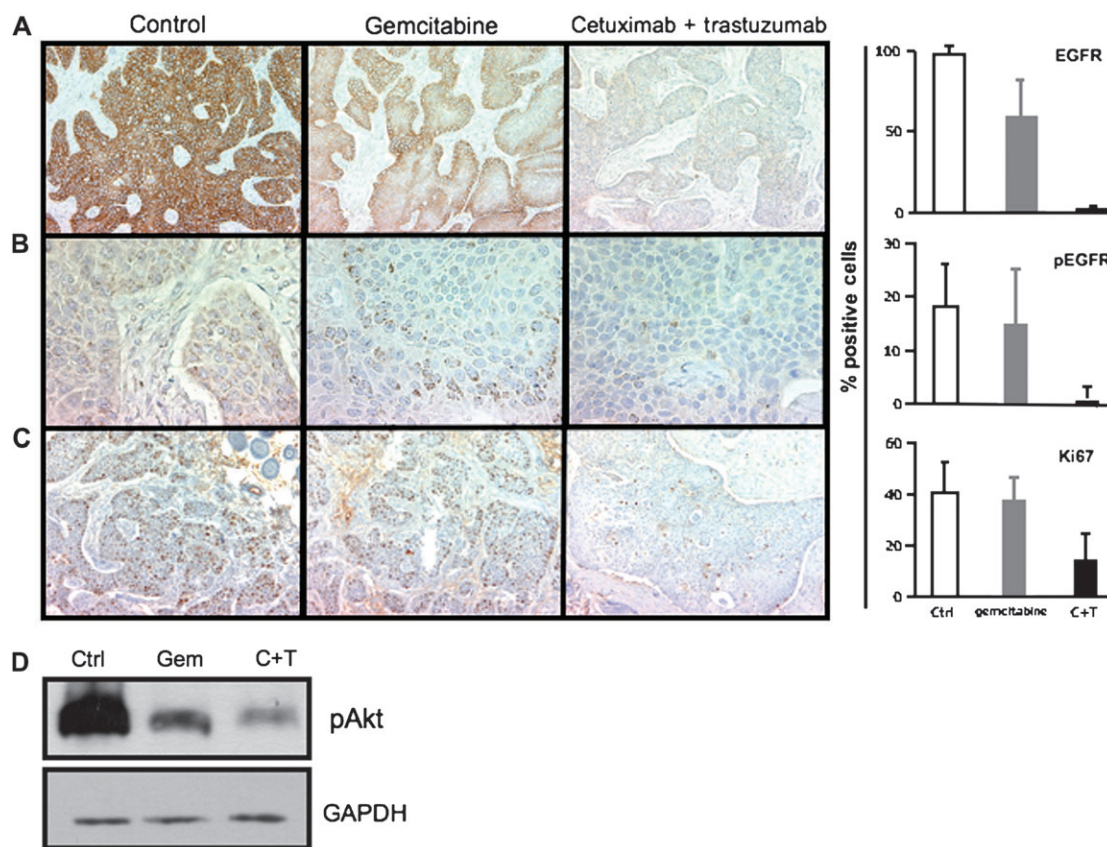


Figure 3. Effect of anti-epidermal growth factor receptor and anti-HER2 monoclonal antibodies (2mAbs) treatment, versus gemcitabine, on the epidermal growth factor receptor (EGFR) expression, EGFR phosphorylation, proliferation index Ki-67 and AKT phosphorylation in BxPC-3 xenografts. Tumors were resected 7 days after the beginning of therapy. Immunohistochemistry shows in (A) EGFR expression (magnification $\times 10$); (B) EGFR phosphorylation (magnification $\times 40$); (C) Ki-67 index (magnification $\times 40$). On the right respective histograms in % positive cells. Western blot analysis shows in (D) the AKT phosphorylation. Glycerinaldehyde-3-phosphate dehydrogenase served as loading control. Cetuximab + trastuzumab (C + T): 2mAbs.

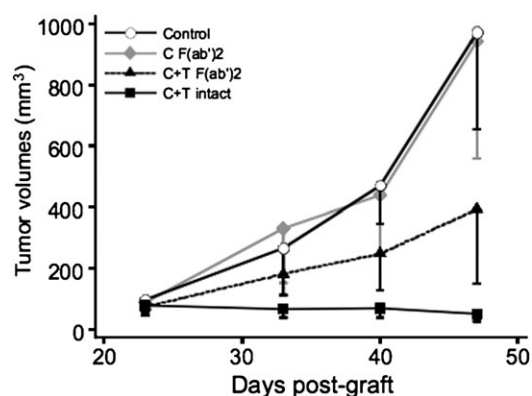


Figure 4. Antitumor effect of the combined treatment with two F(ab')₂ fragments mAbs, compared with two intact mAbs. Mice bearing BxPC-3 pancreatic carcinoma were treated either by coinjection with F(ab')₂ fragments from both cetuximab and trastuzumab or with the intact anti-epidermal growth factor receptor and anti-HER2 monoclonal antibodies or by injection with F(ab')₂ fragments from cetuximab alone or were untreated controls.

growth of a pancreatic carcinoma xenograft, indicating that they can have a therapeutic efficiency independently from the Fc fragment-mediated effector functions and thus due to their

direct binding activity to the HER1 and HER2 receptors. The therapeutic activity of the two intact mAbs was superior to that observed with the two F(ab')₂ fragments, suggesting that the recruitment of effector cells also played a role in the therapeutic efficiency of our 2mAbs treatment. Another explanation for the more efficient therapeutic activity of the two intact mAbs is that due to the property of their Fc fragment to react with FcRn receptor [22], they have a much longer circulating half-life than the F(ab')₂ [23].

Whatever the contribution of the two mentioned antitumor mechanisms, the present results show that the combined use of two anti-EGFR and anti-HER2 mAbs, broadly used in the clinic, can be efficient in the treatment of human pancreatic carcinoma xenografts with low expression of HER2 and in two of them with K-Ras mutations. Most importantly, it demonstrates that the combined anti-EGFR and HER2 therapy is more efficient than the present standard chemotherapy, gemcitabine. In this context, a phase I–II clinical trial evaluating the combined cetuximab and trastuzumab in second-line treatment of pancreatic carcinoma after gemcitabine first-line progression appears justified and promising.

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