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

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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-I) 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-I). 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
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 produced by pepsin 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 Molcaouret, France) were xenografted subcutaneously with BxPC-3 (3.5 × 106), MiaPaCa-2 (5 × 106) and Capan-1 (10 × 106) 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 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: \( V = \frac{4}{3} \pi r^3 \) where \( r \) is the radius and the volumes compared by 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>
<thead>
<tr>
<th>Cell lines</th>
<th>EGFR</th>
<th>HER2</th>
</tr>
</thead>
<tbody>
<tr>
<td>BxPC-3</td>
<td>3+</td>
<td>ND</td>
</tr>
<tr>
<td>MiaPaCa-2</td>
<td>2+</td>
<td>ND</td>
</tr>
<tr>
<td>Capan-1</td>
<td>3+</td>
<td>ND</td>
</tr>
</tbody>
</table>

Ki-67 (MiLB; Dako Corporation, Glostrup, Denmark), EGFR (3C6; Ventana Medical Systems, Ilkirch, France) and pEGFR (tyr 1068, 1H12; Cell Signaling Technology, Beverly, MA) immunoassaying 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.

**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.
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 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...
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.

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

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

*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

### 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.
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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References