Author's response to reviews

Title: Establishment and identification of rabbit model of peritoneal carcinomatosis from gastric cancer

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Author's response to reviews: see over
December 13, 2009
Dear editor:
Thank you for your kind suggestions regarding the revision our manuscript “Establishment and identification of rabbit model of peritoneal carcinomatosis from gastric cancer” (MS: 2225066522825128).

We have made the following language revisions according to editor and reviewers’ comments:

1. First of all, we have thoroughly re-formatted the revised manuscript exactly in accordance with your manuscript template. Therefore, the revised manuscript strictly conforms to your journal style.
2. We have meticulously checked the whole manuscript to ensure that it is free of grammar mistakes.

Regarding comments of reviewer 1, Dr. Andrea McCart, we have made the following revisions:

1. More technical details needed about the surgery. For example how was the stomach exposed?, how did they ensure they were in the submucosal layer?

(1). In the revised text, detailed description of the surgery is provided, along with an added picture showing how the needle was inserted into the submucosal layer of the rabbit stomach for the injection of VX2 cancer cells. The revision is highlighted with green background.

All the animals had overnight fasting before experiment, but water was given ad libitum. After randomization, the animals were anesthetized by ear vein injection of 3% pentobarbital sodium (30 mg/kg). The abdominal skin was cleaned and disinfected. Three approaches were adopted to construct rabbit models of PC, 12 animals for each group. Group A of submucosal tumor cell inoculation: A median incision of 3 cm long was made beginning 2 cm below the xyphoid and the upper abdomen was open. The stomach was exposed and 0.1 mL tumor cells ($5 \times 10^{10}$ vial cells/L) in a 1 mL syringe was injected into the submucosal layer of the stomach (Figure 1), through the serosal layer and the muscle layer, and the injection site was pressed for 1 min to keep the injected tumor cells in place, and the abdomen was closed with a double layer 3-O vicryl interrupted suture. Group B of tumor tissue implantation: The operation procedure was the same as in group A. When the stomach was exposed, a small piece of fresh tumor tissue about 1.0 mm$^3$ was implanted into the greater omentum immediately beneath the gastric antrum, and the wound was closed. Group C of percutaneous injection of tumor cells: After skin preparation, 0.1 mL tumor cells ($5 \times 10^{10}$ vial cells/L) in a 1 mL syringe were injected into the greater omentum, and the injection site was pressed for 1 min. After tumor inoculation, penicillin G at the dose of 100,000 IU/d was intramuscularly injected to each
animal for 3 days. All the animals were given intravenous fluid rehydration with 100 mL of 0.9% normal saline solution.

2. Results. Should discuss reason for death in 3 rabbits.

(2). In the revision, the reasons for the death in 3 rabbits were provided.

Of 36 animals used for PC model construction, the operation was successful in 33 rabbits and 3 animals died 3 days after operation. Of the 3 deaths, 1 rabbit in group C died of diffused peritonitis on day 3 because the percutaneous injection was mistakenly into the small intestine; and 2 rabbits each in groups B and C died of congestive heart failure on day 2 because of fast fluid rehydration. The success rates of different approaches were 100% (12/12) in group A, 91.7% (11/12) in group B and 58.3% (7/12) in group C ($\chi^2=7.047, P<0.031, A$ and $B$ versus $C$).

3. It is not stated when or how animals are euthanised, or criteria for doing so early.

(3). In the section of “animal observation and pathological studies”, we added detailed description of animal euthanasia schedule and gross pathological study.

After model construction, the general health status of rabbits was recorded daily, including food intake, activities, and any abnormalities such as diarrhea and dehydration. The body weight was measured every 3 days and the natural history of the disease progression was recorded. In order to obtain a detailed description of the progressive development of gastric cancer PC, euthanasia was performed on 3 rabbits in group A at the end of weeks 1, 2, 3 and 4, by overdose injection of 3% pentobarbital sodium through the early vein. For animals in groups B and C, euthanasia was also performed when the animals showed obvious signs of distress and waste. Post mortem pathological examinations included gross pathology such as tumor size and distributions; local tumor features of stomach cancer including ulcer formation, obstruction and perforation; special features of peritoneal carcinomatosis such as bloody ascites, discrete or confluent tumor nodules on the peritoneum, cancerous changes in the greater omentum and intestinal obstructions; metastases to major organs such as the liver, adrenal glands, pancreas and the lungs. All the suspected organ tissues were sampled for routine histopathology study with sections stained by hematoxylin and eosin (HE stain).

4. CT scans are performed weekly in weeks 1 to 4, but not clear if this is on all animals or select animals?. Are animals also euthanised at this time. Figure 2 shows an
animals gastric tumour at week 2 but no mention of animals being euthanised or reoperated at this time point.

(4). Whole body CT scans were performed on animals in group A, from weeks 1 to 4, 3 animals at each time point, when the animals were anesthetized. After CT scan, animal euthanasia and pathology study were conducted. Figure 2 shows a picture obtained at pathological study at the end of week 2 after animal euthanasia. The caption for Figure 2 was also revised.

At the end of weeks 1, 2, 3 and 4, 3 animals in group A were anesthetized (the same as above) and subjected to whole body CT scan (SIEMENS senation 16 spiral CT scan, scan parameter: kv: 120 kv; Eff: 180 mAS; slice: 3mm). A gastric tube was inserted into the stomach, through which warm natural saline was infused into the stomach. After the stomach was washed clean, it was filled with 200 mL of warm natural saline, and the CT was performed. Special attention was paid to the chest for any lung metastatic nodules, and to the abdominal cavities for signs of seeding nodules, masses and ascites. The tumor size on the stomach wall was measured and tumor size was calculated as $V=0.5axb^2$, where $a$ represents the maximal diameter and $b$ the minimal diameter.

5. Description of tumour characteristics (page 6) should refer to which group the animals were in, differences seen between groups.

(5). Tumor characteristics were obtained from rabbits in group A. After 4 weeks, all rabbits in groups B and C had advanced peritoneal carcinomatosis. There are no differences between groups.

Tumor characteristics in group A were carefully recorded. One week after inoculation, many small, hard and transparent nodules begin to develop on the greater omentum and the antrum of the stomach. No ascites is found. Two weeks later, nodules on the greater omentum begin to merge into confluent masses with discernable demarcations with the stomach. The mass on the gastric antrum grow bigger and protrudes into the stomach cavity to form typical ulcerative cancer (Figure 2). There is about 5 mL of bloody ascites in the abdominal cavity. Three weeks later, many nodules begin to form in the mesentery and the retroperitoneal peritoneum. The confluent mass on the greater omentum begin to invade the stomach wall and encase the stomach (Figure 3). Often the stomach mass and the greater omentum mass are merged into one big tumor block, which can not be separated. Bloody ascites could reach as much as 100 mL. There is also some fluid accumulation in the pericardium. Many nodules are seen on the abdominal wall. No nodules are seen in the lungs. Four weeks later, many tumor nodules are found in the lungs, small intestine, transverse colon, bladder and adrenal glands, and the liver. Many hard and whitish nodules are seen on the liver and the abdominal wall is totally invaded by the tumor. Tumors in groups B and C showed the same growth characters, except that
animals in group C did not develop ulcerative gastric cancer.

6. Histopathological characteristics: is this 2 animals per group? or just 2 animals from group 1. if only group 1 why not other groups? what were differences seen between groups?

(6). We studied the histological features of the tumor tissue from rabbits of all the three groups, and found similar histopathological characteristics. There are no differences among the three groups.

Two weeks after gastric antrum inoculation of VX2 tumor cells, 2 rabbits are sacrificed after euthanasia to allow a detailed histopathological evaluation of the tumor in the stomach. The tumor is on the greater curvature side of the gastric antrum, penetrating the mucosal layer to form an ulcer. Microscopic view could find cancer nests penetrating the whole stomach wall, with typical invasion into the muscle layer and the gastric glands (Figure 4A). The tumor cells are round, oval or atypical morphology with many pathological mitotic figures (Figure 4B). There are also conspicuous lymphocytes, plasma cells and other inflammatory cells infiltration.

Histopathological studies of tumor tissues from rabbits of other groups and time points showed the same feature.

7. It would be very helpful to have a table characterizing the differences between groups such as: ct results, histopathology, tumour distribution etc.

(7). We summarized the key features of three different approaches in a new table.

<table>
<thead>
<tr>
<th>TABLE 2. Tumor characteristics of three different inoculation approaches</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group A</strong></td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Technical feature</td>
</tr>
<tr>
<td>Success rate</td>
</tr>
<tr>
<td>Major pathological events</td>
</tr>
</tbody>
</table>
obstruction, renal failure

<table>
<thead>
<tr>
<th>CT scan</th>
<th>Lung metastasis, gastric tumor mass, PC, ascites</th>
<th>Gastric tumor mass, PC, ascites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gross pathology</td>
<td>Ulcerative gastric cancer with PC, ascites</td>
<td>Ulcerative gastric PC without gastric cancer with PC, ulcer, ascites</td>
</tr>
<tr>
<td>Histopathology</td>
<td>Penetrating growth of cancer cell nests invading surrounding structures, tumor necrosis in the central zone of the tumor mass</td>
<td></td>
</tr>
<tr>
<td>Advantages</td>
<td>Most resemble clinical gastric cancer with PC</td>
<td>Technically less difficult</td>
</tr>
<tr>
<td>Disadvantages</td>
<td>Technically difficult</td>
<td>Not exactly mimic gastric cancer with PC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mistaken injection into the intestines</td>
</tr>
</tbody>
</table>

8. In discussion, again authors need to differentiate between their groups. Which was the most like gastric cancer with peritoneal deposits? Was one method more aggressive that the others? Why did group c only have a 58% success rate? Need to discuss the limitations of this model as well ie squamous cell cancer histology may differ from adeno ca.

(8). We have studied the comments by Jens Hartmann, reviewer 3, who suggested one mice models of PC from melanoma cell line and one rat model of PC from colon cancer cell line. The advantages and limitations of our model, compared with those reported in literature, are discussed in detail. We also elaborated on the possible mechanisms by which why submucosal injection of cancer cells could result in more aggressive tumor, and why this approach is more appropriate. The differences between squamous carcinoma and adenocarcinoma are also discussed in the revision.

PC represents a serious clinical challenge in the treatment of gastrointestinal and gynecological cancers. In gastric cancer, PC is a frequent event even in the early phase of the disease, with 15% to 50% or more having PC at the surgical exploration, especially when there is serosal involvement by the tumor [12-14].
Even after curative resection of gastric cancer, PC remains the major problem of postoperative recurrence. A large scale, single center and long term Korean study in 500 gastric cancer patients treated by standardized radical gastrectomy and lymphadectomy, found that within 5 years post gastrectomy, PC is the most frequent pattern (51.7%) of cancer recurrence [15]. Another large scale, randomized control prospective study in Japan also found peritoneal recurrence is the most frequent event (15.8%) at 3 years in 530 patients treated with highly standardized curative gastrectomy [16]. A prospective Italian study in 200 patients found that, at the mean follow-up of 42.3 months, PC accounted for 32.9% of recurrence [17]. Another large Italian study with 441 patients with gastric cancer showed 17% PC recurrence at the median follow-up of 48 months [18]. Therefore, synchronous and metachronous PC is the most important problem of gastric cancer recurrence and metastasis. Such gastric PC is associated with poor prognosis with median survival ranging from 1-1.6 months [19,20] to 3.1-9 months [1,13]. As is rightly stated, the risk of peritoneal recurrence of gastric cancer is particularly high in patients with diffuse-mixed tumors and infiltration of the serosa, against which surgery alone, no matter how radical, can offer little possibility of a cure [18]. Therefore, new comprehensive treatment strategies are required.

Clinical studies suggest that CRS plus HIPEC could achieve good efficacy in selected patients with PC. To our knowledge there are 4 institutional studies on CRS plus HIPEC in patients with gastric cancer, 2 retrospective (42 and 26 patients, respectively) [3, 21], 1 prospective (49 patients) [4] and 1 comparative non-randomized (34 patients) studies [22]. The median survival ranged from 6.6 months [22], 8 months [21] to 10-11 months [3,4], and the 5-year survival ranged from 6% (3,26) to 16% according to Glehen et al [4]. Such procedures are risky and resources consuming. As many experts argue, CRS plus HIPEC remains an approach less mature and to be reserved for centers which are familiar with the procedure to maintain an acceptable operative risk and select the patients who presumably can have the best benefit-to-risk option [12]. These clinical trials are based on non-homogenous and non-standardized groups of patients. In order to more objectively evaluate such treatment, it is worthwhile to study this treatment modality under experimental conditions.

Many animal models of PC have been established, including nude mice models and rat models [23,24]. 1. Braumann C, Stuhldreier B, Bobrich E, Menenakos C, Rogalla S, Jacobi CA. High doses of taurolidine inhibit advanced intraperitoneal tumor growth in rats. J Surg Res. 2005 Nov;129(1):129-35. 2. Braumann C, Jacobi CA, Rogalla S, Menenakos C, Fuehrer K, Trefzer U, Hofmann M. The tumor suppressive reagent taurolidine inhibits growth of malignant melanoma--a mouse model. J Surg Res. 2007.]. In most of these animal models, cancer cells are injected directly into the peritoneum, which will result in widespread PC in due time [25-28]. All these small animal models are only suitable for HIPEC alone because the small body size and limited blood supply cannot stand major surgical interventions. The establishment of large animal model of PC from gastric cancer is of considerable practical value. It could help experimental studies testing the combination of CRS and HIPEC.
VX2 carcinoma is a rabbit tumor of epithelial origin, established from a virus-induced papilloma by Rous and coworkers [29]. Characterized by rapid tumor growth and early metastasis, this tumor is extremely malignant and can be allogeneously transplanted almost anywhere in rabbits. Although VX2 is a squamous cell carcinoma model, which may be different from adenocarcinoma, it has been used in a variety of experimental studies on head and neck cancer [30-33], lung cancer [34], esophageal cancer [35,36], breast cancer [37], gastric cancer [38,39], liver cancer [40,41], colon cancer [42,43], kidney cancer [44-46], bladder cancer [47], bone tumor [48] and simple peritoneal carcinomatosis [49]. In this study we constructed a rabbit model of gastric cancer with PC. We tested three different approaches, tumor cells injection into the submucosal layer of the stomach, tumor tissue implantation into the greater omentum beneath the gastric antrum, and tumor cells injection into the peritoneal cavity. Our results demonstrated that the orthotopic inoculation of tumor cells into the stomach is the most appropriate method, resulting in typical ulcerative gastric cancer and progressive PC, all features similar to the clinico-pathologic progression of gastric cancer patients. In comparison, percutaneous injection of cancer cells into the abdominal cavity could result in intestinal injury by mistake. No ulcerative gastric cancer could be induced, and the tumor take rate is less optimal. These results support the notion that orthotopic tumor model is preferred for the study of tumor biological behaviors and interventions [50]. The key to developing such a successful model is suitable suspension of cancer cells directly injected under sterile conditions. The natural history of this model is about 4 weeks, including a subclinical stage for the first week, clinical stage for the second week, accelerated PC stage in the third week and pulmonary metastases to terminal stage in the fourth week. Typical PC features are peritoneal cancer nodules of various sizes throughout the whole abdominal cavity, “omentum cake”, intestinal obstruction and bloody ascites. Compared with our previous nude mice model of PC [7], these features are more prominent and can be more objectively identified.

Based on the progression features of this model, it could be seen that the end of the first week could be considered at the early PC stage, while the end of the second week as the advanced PC stage. Therefore, different treatment approaches could be designed more specifically to target either early PC or advanced PC.

9. Conclusion: “A rabbit model of gastric cancer with PC has been established”.
Which group or groups does this refer to?

(9) We have revised the conclusion as the following.

A rabbit model of gastric cancer with PC has been established by injecting VX2 cancer cells into the submucosal layer of the rabbit stomach. The model is characterized by typical ulcerative gastric cancer with progressive PC. Compared with other small animal PC models, this rabbit model of PC is more suitable for surgical intervention because of the big body size, for the evaluation of comprehensive treatment approaches against PC because of the more typical gastric PC features.
Minor essential revisions:

10. While the manuscript is very understandable it needs a thorough editing for grammatical mistakes. It should be sent to an english speaking person for review. (Too many to list here)

(10). We have read the corrected all the grammatical mistakes, and made the text more understandable.

11. Needs a reference for and description of the rabbit VX2 model which is actually a transplantable rabbit squamous cell cancer. (this is referenced in discussion, should be moved up to methods section).

(11). Following the reviewer’s suggestion, we have provided the brief history of VX2 tumor in the method section.

Rabbit VX2 carcinoma was used for the construction of gastric cancer with PC in this study. VX2 tumor is a transplantable rabbit squamous cell carcinoma, characterized by rapid tumor growth and early metastasis, established from a virus-induced papilloma by Rous and coworkers [29]. The tumor was maintained by successive in vivo transplantation into the hind limb of a carrier rabbit, with 2 rabbits used for each generation of passage. Tumor cells suspension (5×10^10 cells/L, 0.1 ml) was injected subcutaneously into the hind leg of each carrier rabbit and the tumor grew for 3 weeks.

12. the description of the three groups in the methods does not match the description given in the abstract. In the abstract it stated 6 animals per group received a cell suspension and 6 received tissue But in the methods only group B received tissue. This needs to be completely clarified.

(12).In the revised text, we have provided detailed description of the animal grouping, 12 rabbits in each group.

13. statistical analysis describes nude mice. this should be clarified.

(13).In the revision, the statistical analysis describes rabbits, not nude mice.

14. Figures:
1. is this all groups. are they all the same?
2. which group is this from?
3,4,5: please add group and time from tumour injection?

(14).In revised Figure 1, we have provided the body weight change curves for groups A, B and C, respectively.
Figure 2 is from group A.
In Figures 3, 4 and 5, information on group and time is provided.

**Regarding comments of reviewer 1, Dr. Sachio Fushida, we have made the following response:**

Since the reviewer did not raise any concrete remarks, and our academic interest is not appeal to the reviewer, we could not make any revision based on the reviewers comments.

**Regarding comments of reviewer 3, Dr. Jens Hartmann, we have made the following revisions:**

1. Language has been checked meticulously, and any ambiguity or grammar mistakes are corrected.
2. In the Background and Methods sections, the reviewer’s suggestions have been accepted. Two references cited by the reviewer have been cited and commented in the text.
3. In the discussion and conclusions sections, careful revision has been made as stated above.

We hope these point-to-point response to your kind suggestions could make the manuscript more clear and up to your standard.

Thank you again for your kind assistance, and also special thanks to reviewers for their time input and valuable suggestions.

With kind regards,
Yours Sincerely,

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