Author's response to reviews

Title: The associated expression of Maspin and Bax proteins as a potential prognostic factor in intrahepatic cholangiocarcinoma

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Author's response to reviews: see over
After carefully reading the comments of the reviewers and on behalf of our colleagues Soliani, Desenzani and Crafa, we resubmit a revised version of the manuscript “The associated expression of Maspin and Bax proteins as a potential prognostic factor in intrahepatic cholangiocarcinoma”.

As suggested by the editor we discuss (Discussion section, pag 21) how the number of patients enrolled in this study may be a limitation of the conclusion that can be drawn. However, our cohort represent a large single institution series given the rarity of this tumor.

As required by the Editor, we have now indicated in the revised version (Sample source paragraph of the Methods section) that “the study was conducted in accordance with the tenets of the Declaration of Helsinki. Following the indication of Italian DLgs no. 196/03 (Codex on Privacy), every precaution has been taken to respect the privacy of the subject and the confidentiality of the patient’s information. A written consent to use stored tissue was obtained from still alive patients”.

When this study started one year ago the local “Research Ethics Committee of the Medical Faculty” (University of Parma, chairman the Dean of the Faculty) did not require a mandatory application for approval in the case of researches based on retrospective (but not genetic) analysis of specimens previously used for pathological evaluation and already stored in the istopathology archive of the Pathology department. However, the principles of the Declaration of Helsinki were mandatory and must be followed.

The changes made and our answers to the reviewer’s requests are below listed. Due to the changes made in according to reviewers requests, there were changes in the text, numbers of figures. Some new references were also added.

The changes that we have made in response to the criticisms have undoubtedly improved the paper and hope that this revised version will be deemed acceptable.

The changes made to the original are indicated in red color in the revised manuscript:

**First reviewer:**

1. The authors proposed that the related expression of maspin and Bax may influence the susceptibility of tumor cholangiocytes to apoptosis and therefore would be involved in delaying IHCCA progression. To confirm this finding, staining of apoptotic cell should be done (for example TUNEL).

In agreement with the reviewer’s suggestion we have analyzed in our samples the occurrence of apoptotic cells. After a review of literature (J Pathol. 2003;199:221-8, Endocrinology. 2002; 143:1495-501, Anat Rec A Discov Mol Cell Evol Biol. 2003; 272:503-13), we decided to use antibody directed against a cleavage product of caspase-3 (a downstream effector caspase) to highlight the apoptotic cells. This suggestion, in our opinion, surely improved the scientific message of the paper.

2. To analyze cholangiocarcinoma, why the authors did select the maspin gene from a pool of mRNA sequence identified by in silico screening?
As indicated now in the *Introduction section*, we focused our attention on those over-expressed genes known to counteract or delay tumor progression. Among these we selected the Maspin gene whose protein product belongs to the family of protease inhibitors.

**Second reviewer:**

- **Study**

  1. *How did authors define their cohort of cholangiocarcinoma?*

     In the *Patients* paragraph of the *Methods section* we have now indicated all the clinical details to define our cohort of patients enrolled in this study.

  2. *The manuscript tends to use CC at one place and then IHCC at other places in the manuscript.*

     The conflicting meaning between CCA and IHCC disease has been amended. Therefore, except in the first paragraph of the *Introduction section*, throughout the whole paper the term “IHCCA“ has been used.

  3. *Authors have written that Maspin expression causes either increase or decrease in the tumor volume. Did they mean association? Similar conceptual errors are noted throughout the manuscript.*

     The Result section has been modified to improve clarity. However, we wished to stress that the associated expression of Maspin and Bax determines a significative reduction of tumor mass.

- **Major Compulsory Revision**

  1. *Authors must change english and provide conceptual clarities and consistency in the text of the manuscript.*

     An english native-language colleague reviewed and amended the manuscript.

  2. *In the Method section: Authors do not provide detailed information on how patients were selected or not included in the study and the reasons for it.*

     In the Method section, a detailed information on how the patients were selected is now provided. In particular, the epidemiological data, retrieved from Parma Registry Tumors (*Divisione di Oncologia Medica, Azienda Ospedaliera di Parma*), showed in the last ten years a sharp increment of IHCCA in our province from 1-2 cases/year in 1994 to 9-12 cases/year in 2002-2004.

  3. *Furthermore, it would add value to their descriptions if they include more details of how the tissue sections were selected and which sample types were evaluated (e.g., more differentiated areas or poorly differentiated areas).*

     In the *Sample Sources* paragraph of the *Methods section* informations of how the tissue section were selected and which sample types were evaluated are now indicated in detail.
4. Introducing the review of control group (adjacent normal/reactive ductal epithelium) also would help understand the value of analysis that was performed on this valuable resource.

In the Immunohistochemistry paragraph of the Methods section reviews of control groups are now included. In particular, adjacent normal and/or dysplastic areas were selected as internal controls.

5. **Authors have not provided any detail on how Bax was evaluated.**

The evaluation of Bax is now provided in Quantification of expression paragraph of Methods section.

6. **In addition, it would be useful to describe which part of the tumor was analyzed for micro vessel density and whether new vessels or thick vessels at the periphery were analyzed.**

Detailed description of microvessel evaluation is now included in the Evaluation of microvessel density paragraph of Methods section.

7. **They also need to provide better descriptions of how they evaluated each marker, how did they correlate it with controls and the basis for using the cut off values.**

A new paragraph called “Immunostaining of Maspin, Bax and cleaved caspase-3” in the Result section is now inserted to provided better descriptions about each marker and method for using the cut off value.

8. **Better figures are needed to corroborate the manuscript. It appears that there is a green hue noted in these photomicrographs.**

A new set of photomicrographs is now provided.

Yours sincerely,

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