Could antiplatelet therapy prevent hepatocellular carcinoma? 7 Tesla liver magnetic resonance imaging study in a mouse model of hbv-related chronic hepatitis

Poster No.: B-0239
Congress: ECR 2013
Type: Scientific Paper
Authors: P. Marra, A. Esposito, G. Sitia, A. Palmisano, T. Canu, F. De Cobelli, L. G. Guidotti, A. Del Maschio; Milan/IT
Keywords: Liver, Oncology, MR, Image manipulation / Reconstruction, Efficacy studies, Contrast agent-intravenous, Computer Applications-3D, Cirrhosis, Infection, Pathology
DOI: 10.1594/ecr2013/B-0239

Any information contained in this pdf file is automatically generated from digital material submitted to EPOS by third parties in the form of scientific presentations. References to any names, marks, products, or services of third parties or hypertext links to third-party sites or information are provided solely as a convenience to you and do not in any way constitute or imply ECR's endorsement, sponsorship or recommendation of the third party, information, product or service. ECR is not responsible for the content of these pages and does not make any representations regarding the content or accuracy of material in this file.

As per copyright regulations, any unauthorised use of the material or parts thereof as well as commercial reproduction or multiple distribution by any traditional or electronically based reproduction/publication method ist strictly prohibited.

You agree to defend, indemnify, and hold ECR harmless from and against any and all claims, damages, costs, and expenses, including attorneys' fees, arising from or related to your use of these pages.

Please note: Links to movies, ppt slideshows and any other multimedia files are not available in the pdf version of presentations.

www.myESR.org
Hepatitis B virus (HBV) chronically infects more than 350,000,000 people worldwide and in most cases antiviral therapy fails to eradicate the infection\textsuperscript{1,2}. Moreover chronic liver inflammation is caused by a dysfunctional T cell response that increases the risk of developing cirrhosis, liver failure and hepatocellular carcinoma (HCC)\textsuperscript{3}.

Recently it has been shown that in murine models of acute viral hepatitis the hepatic recruitment of virus-specific cytotoxic T lymphocytes (CTLs) is mediated by platelets and antiplatelet therapy can reduce liver immunopathology\textsuperscript{4,5}. Based on these findings we hypothesized that also in chronic hepatitis B (CHB) platelets contribute to the hepatic accumulation of HBsAg-specific CTLs. So, a prophylactic treatment with aspirin and clopidogrel may limit liver inflammation and prevent the development of fibrosis and hepatocellular carcinoma.

The aim of this work was to monitor the development of HCC by preclinical 7T liver Magnetic Resonance Imaging (MRI) in a mouse model of immune-mediated CHB in order to evaluate the eventual efficacy of an experimental therapy with low doses of aspirin and clopidogrel in the prevention of hepatocarcinogenesis.

Data from longitudinal volumetric evaluation at MRI were also compared with biochemical analyses, histopathology and survival curves.
Images for this section:

**Fig. 1:** Confocal microscopy showing interaction between platelets (red) and an HBsAg-specific cytotoxic T lymphocyte (green) inside a liver sinusoid (blue).

© Experimental imaging centre, San Raffaele scientific institute, Milan/ Italy
The mouse model of chronic hepatitis B relies on 107-5 transgenic mice (inbred B10D2) in which 100% of hepatocytes express nontoxic quantities of the HBV large, middle and small envelope proteins\textsuperscript{6}. 107-5 mice are immunologically tolerant to HBsAg and develop chronic immune-mediate hepatitis only after bone marrow transplantation and transfer of spleen cells obtained from non-transgenic mice previously primed with HBsAg (Fig. 2). 8 to 10 months after disease induction the liver shows typical signs of CHB such as regenerative, hyperplastic and dysplastic nodules, fibrosis and hepatomas.

220 mice with chronic hepatitis were randomized to receive a placebo (Vehicle group = 110 mice) or an experimental therapy with aspirin and clopidogrel (Asp/Clo group = 110 mice). 220 healthy mice were divided into two control groups (Sham group = 110 just irradiated mice; CTRL group = un-manipulated mice) to consider the eventual carcinogenicity of the immunological procedure or a spontaneous development of hepatomas (Fig. 3).

20 mice per group (per time point) underwent in vivo and ex vivo liver MRI at 9 and 15 months after disease induction with a 30-cm-bore 7T magnetic resonance scanner (BioSpec 70/30 USR, Paravision 5.0; Bruker) to diagnose HCC, determine HCC volume and generate 3D liver reconstructions. In vivo MRI relied on the acquisition of fat-sat T2 weighted turbo-RARE and T1 weighted RARE sequences after the intravenous administration of Gd-EOB-DTPA (35µg/g of body weight, Primovist; Bayer Schering Pharma). Livers explanted were fixed in formaldehyde and re-examined with ex vivo liver MRI acquiring an extra 3D T1 weighted FLASH sequence. The specific sequence parameters for T2w or T1w acquisition, respectively were as follows: T2w [Turbo rapid acquisition relaxation enhanced T2 sequence]: (TR) = 2744 ms, (TE) = 33 ms, voxel size = 0.097 x 0.117 x 0.8 mm; T1w [RARE T1 sequence]: (TR) = 866 ms, (TE) = 8.6 ms, voxel size = 0.089 x 0.136 x 0.8 mm. All in vivo MRI sequences were analyzed with advanced image segmentation software (Mipav, 5.3.4 version, National Institutes of Health) and liver lesions were manually identified based on hypointensity on Primovist-enhanced T1w images and hyperintensity on T2w images on each slice.

Ex vivo MRI images were precious to generate 3D reconstructions (volumetric rendering) after manual segmentation of livers and tumors and they were also directly compared with histopathology at autopsy.

Other samples of 20 mice per group underwent sacrifice at different time points for histological analyses concerning liver immunopathogenesis, hepatocellular regeneration and fibrosis deposition.

20 mice per group were used to build Kaplan-Meier survival curves.
Fig. 2: Steps of the immunological manipulation needed to induce chronic immune-mediated hepatitis in 107-5 mice.

© Immunopathology unit, San Raffaele scientific institute, Milan/Italy

Fig. 3: Four different groups of mice enrolled in the study: Sham and CTRL mice underwent only partial immunological manipulation to rule out a predisposition to hepatocellular carcinoma both before and after irradiation. Transplanted mice were randomized to receive a placebo (Vehicle) or the experimental therapy (Asp/Clo) at the doses shown.

© Immunopathology unit, San Raffaele scientific institute, Milan/Italy
Results

HCC diagnosis

At in vivo MRI it was possible to identify 100% of HCCs with a diameter of 0.5 mm and larger and they were correctly differentiated from non-malignant lesions (cysts, angiomas, regenerative nodules, low grade dysplastic nodules) based on their features on T2w and post-contrast T1w images (see Methods). All suspect nodules identified at MRI (Fig. 4) were confirmed at autopsy as well-differentiated HCCs characterized by trabecular cords of neoplastic hepatocytes that were not infiltrated by inflammatory cells and expressed no HBsAg (in contrast to hepatocytes from the surrounding noncancerous liver parenchyma).

Antiplatelet therapy prevents or delays the development of HCC

At both 9 and 15 months the treatment with aspirin and clopidogrel was associated with a significant reduction of HCC incidence (Fig. 6): none of the CTRL or the Sham group mice developed HCC; in contrast, at the same time points, respectively 30% and 65% of the Vehicle group mice displayed HCCs while prevalence of HCC-bearing mice in Asp/Clo group was just 5% at 9 months and 20% at 15 months (Fig. 7).

In mice that developed HCC total tumor volume was calculated and expressed as the ratio "total tumor volume/total liver volume" (a parameter encompassing both the number and the size of tumors per liver). In mice treated with aspirin and clopidogrel this parameter was significantly smaller both at 9 and at 15 months than in Vehicle mice (Fig.8) suggesting that the experimental therapy delayed HCC development. Figure 9 and 10 show two representative livers of Vehicle and Asp/Clo mice at 15 months.

Antiplatelet therapy reduces the severity of liver fibrosis

Treatment with Asp/Clo was associated with a reduction of liver immunopathology, inflammatory cells infiltration and hepatocellular regeneration (data not shown). The amelioration of the CHB scenario due to antiplatelet therapy leaded also to a reduction of collagen deposition: although none of the mice became cirrhotic the severity of liver fibrosis was significantly reduced in Asp/Clo mice (Fig. 11).

Antiplatelet therapy improves survival
At day 510, 15/20 mice of the Vehicle group had died while 16/20 mice of the Asp/Clo group were alive in good health conditions. By day 520, all remaining mice of the Vehicle group were euthanized for ethical reasons. With the exception of a single Asp/Clo-treated mouse that died by day 550, remaining Asp/Clo animals survived until day 600, when they were euthanized (Fig. 12). At autopsy, less than 50% of mice of the Asp/Clo group presented few small HCCs while all mice of the Vehicle group displayed numerous large tumors.
Fig. 4: Two small nodules identified at in vivo MRI at 9 months after CHB induction. These lesions were confirmed as well-differentiated HCC at postmortem histopathology.

© Radiology, Ospedale San Raffaele - Milan/IT
**Fig. 5:** A mouse liver with signs of diffuse hepatocellular regeneration: note the numerous hyperplastic nodules that are characterized by hypointensity on T2w images and iso-hyperintensity on post-contrast T1w images. The benign nature of these nodules was confirmed at histology.

© Radiology, Ospedale San Raffaele - Milan/IT
Fig. 6: This axial post-contrast T1w image shows an HCC lesion with "nodule-in-nodule" appearance. This feature is probably typical of HCCs that develop inside regenerative nodules where the process of DNA replication is particularly active.

© Radiology, Ospedale San Raffaele - Milan/IT
**Fig. 7:** Incidence of HCC was significantly smaller in Asp/Clo-treated mice both at 9 months (p < 0.001) and at 15 months (p < 0.0001) than in Vehicle mice (20 mice per group per time point).

© Immunopathology unit, San Raffaele scientific institute, Milan/ Italy
Fig. 8: Mass of HCC was 3.8-fold and 5.4-fold smaller in Asp/Clo-treated mice at both time points indicated (p < 0.001) than in Vehicle mice (20 mice per group per time point).

© Immunopathology unit, San Raffaele scientific institute, Milan/ Italy
**Fig. 9:** Shortly after in vivo MRI mice were sacrificed and livers explanted were fixed in Zn-formalin to undergo ex vivo MRI and histopathologic evaluation. Ex vivo MRI 3D T1w sequences were applied for volume rendering reconstructions (on the right) where normal parenchima is represented in yellow while HCCs in red.

© Radiology, Ospedale San Raffaele - Milan/IT

**Fig. 10:** Two livers of HCC-bearing mice at 15 months representative of the indicated groups at in vivo post contrast MRI with their respective 3D reconstructions. At this time point a typical liver of a placebo-treated mouse (on the left) appeared enlarged with irregular profiles and presented numerous and large HCCs (see video at figure 13). In
contrast a typical liver from the Asp/Clo group (on the right) was normal in size and shape and developed few HCCs (see video at figure 14).

© Radiology, Ospedale San Raffaele - Milan/IT

**Fig. 11:** Sirius red stain for quantification of collagen deposition. At 9 months and 15 months mice from Asp/Clo group had a significantly lower extent of liver fibrosis than Vehicle mice (20 mice per group per time point).

© Immunopathology unit, San Raffaele scientific institute, Milan/ Italy
Fig. 12: Kaplan-Meier survival curves of mice of indicated group. Asp/Clo treatment in CHB mice was associated with a significant survival improvement (p < 0.0001), (20 mice per group).

© Immunopathology unit, San Raffaele scientific institute, Milan/ Italy
Fig. 13

© Radiology, Ospedale San Raffaele - Milan/IT
Fig. 14

© Radiology, Ospedale San Raffaele - Milan/IT
Conclusion

Antiplatelet therapy with aspirin and clopidogrel is commonly used for cardiovascular prevention and in many cases its assumption may be protracted all lifelong. In recent years lots of patient chronically assuming aspirin have been studied to eventually confirm the anti-neoplastic effect of this compound, originally suggested by preclinical studies about gastrointestinal tumors in mice and rats\(^7\). A recent meta-analysis retrospectively investigated the efficacy of aspirin in the prevention of various types of cancer in patients assuming antiplatelet therapy for several years: it was interestingly shown that aspirin can reduce the incidence of prostate, lung, colorectal and other cancer with adenocarcinoma differentiation, even if the precise mechanism of action is still unknown\(^8\).

To our knowledge this is the first time that a preclinical study directly demonstrates the efficacy of antiplatelet therapy in the prevention of hepatocellular carcinoma: in this model of chronic hepatitis B, aspirin and clopidogrel probably act inhibiting the hepatic accumulation of virus-specific cytotoxic lymphocytes, thus reducing chronic liver necroinflammatory disease which is responsible of DNA damage and hepatocarcinogenesis\(^9\).

Preclinical 7T MRI contributed to demonstrate the efficacy of the experimental Asp/Clo therapy: this imaging modality was useful to diagnose and monitor HCC development non-invasively in a transgenic mouse model of chronic hepatitis B. The reduced incidence and the delayed development of HCC in Asp/Clo group mice at MRI coincided with an effective reduction of liver immunopathology at histology and with a significant improvement of survival.

If these results will be confirmed in clinical trials, antiplatelet therapy with aspirin and clopidogrel would be used for cirrhosis and hepatocellular carcinoma prevention in patients chronically infected with hepatitis B virus.
References


Personal Information

Paolo Marra, Antonio Esposito, Anna Palmisano, Tamara Canu, Francesco De Cobelli, and Alessandro Del Maschio;

- Preclinical MR and US Facility, Experimental Imaging Center, San Raffaele Scientific Institute, Milan, Italy.

- Department of Radiology, San Raffaele University Hospital, Milan, Italy.

Giovanni Sitia and Luca G. Guidotti;

- Immunopathology unit, San Raffaele Scientific Institute, Milan, Italy.

Corresposne to:

- Paolo Marra: p.marra@studenti.unisr.it

- Antonio Esposito: esposito.antonio@unisr.it