Allegato/Annex B

PROGETTO DI RICERCA / RESEARCH PROJECT
(max 5 pagine / max 5 pages)

Cognome/Surname	Celsa
Nome / Name	Ciro
Titolo del progetto / Project title	Risk profiling of hepatocellular carcinoma occurrence and recurrence after eradication of HCV with direct-acting antivirals.
Corso di dottorato / PhD	Oncologia e chirurgia sperimentali
Firma del candidato/ Applicant's signature	

1 - Sommario / Abstract

Eradication of hepatitis C virus (HCV) by direct-acting antivirals (DAAs) reduces but does not eliminate the risk of hepatocellular carcinoma (HCC) occurrence and recurrence in patients with cirrhosis. Predictive biomarkers of risk for HCC still remain a highly unmet medical need. The aim of this project is to identify clinical, genetic and epigenetic risk factors for HCC occurrence and recurrence in patients with HCV cirrhosis who achieved DAA-induced sustained virologic response (SVR). All consecutive patients with HCV cirrhosis who obtained DAA-induced SVR will be evaluated for HCC occurrence and HCC recurrence by Kaplan-Meier curves. Variables associated with HCC will be analyzed by Cox regression analysis. Genotyping of selected single nucleotide polymorphisms will be carried out on blood samples. Gene expression will be performed on liver tissue samples using real time PCR and western blot. Circulating-free DNA analysis will be performed on plasma and serum samples and ultra-deep DNA sequencing will be performed for a panel of selected genes. Epigenetic modifications will be assessed on liver tissue samples.
The combination of clinical, genetic and epigenetic variables could identify personalized risk profiles for HCC occurrence and recurrence in patients who obtained SVR after DAAs.
Improvement in prediction of HCC after SVR could result in earlier detection, personalization of surveillance programs and improvement of patients outcomes.

2 - Descrizione del progetto / Project

Background

Hepatocellular carcinoma (HCC) is the most frequent primary liver cancer. Worldwide, HCC represents the sixth most common neoplasm and the fourth most common cause of cancer-related death, with an incidence that is expected to increase in the future.[1-2] According to the annual projections of the World Health Organization, more than 1 million patients will die from HCC in 2030.[3]

The development of HCC is closely related to the presence of chronic liver disease, mostly as a result of hepatitis B or C virus (HBV or HCV) infection, alcohol abuse or nonalcoholic fatty liver disease (NAFLD). The risk varies according to etiology, geographic area, sex, age, and severity of liver disease, in terms of portal hypertension and stage of cirrhosis (compensated or decompensated).[4] HCC represents the leading cause of death in patients with compensated cirrhosis and the underlying chronic liver disease has a significant impact on the feasibility, efficacy and safety of treatments for HCC.

HCV infection represents an independent risk factor for the development of HCC,[5] although HCC rarely occurs in those who do not have advanced fibrosis. Sustained virologic response (SVR) obtained with both interferon[6] or direct-acting antiviral agents (DAAs)[7] reduces the risk of incident hepatocellular carcinoma and DAA therapy improves survival through a protective effect on liver decompensation in patients with previous successfully treated early HCC, without affecting the risk

of HCC recurrence.[8] The implementations in the prevention and the management of viral chronic liver diseases are changing the landscape of HCC: on one side, NAFLD, that is frequently associated with metabolic syndrome, obesity and type 2 diabetes, is becoming the most frequent cause of HCC in Western countries[9], while on the other side, the development of HCC after SVR remains a critical issue even in the DAA-era. When a change of state occurs from a viremic phase to SVR, the determination of the HCC risk profiling must take into account the presence of residual risk factors for HCC, such as the severity of portal hypertension, the prognostic stage of cirrhosis and the presence of metabolic comorbidities (i.e. type 2 diabetes and obesity).
European guidelines recommend abdominal ultrasonography (US) every six months to all patients with cirrhosis, and every three months in those who underwent to a curative treatment,[10] but they do not take into account that individual risk is highly heterogeneous according to liver disease severity, to different etiologies or to response to etiologic treatments (i.e. SVR status or HBV-DNA suppression).[11] Clinical, biochemical, histological and virologic features have been identified as potential pre- or post-treatment risk factors for developing HCC occurrence and recurrence after SVR in patients with HCV chronic liver disease, i.e. male sex, advanced age, alcohol intake, presence of type 2 diabetes, advanced fibrosis stage, lower platelet count and albumin level, α-fetoprotein (AFP), higher aspartate aminotransferase-to-platelet ratio index (APRI), HCV genotype 3.[7,12-15] However, their usefulness in the prediction of the risk for development of HCC in the single patient is often limited and predictive biomarker models represent an urgent unmet medical need to enable HCC surveillance for early detection and to predict the prognosis. Recently, genome-wide association studies (GWAS) identified single nucleotide polymorphisms (SNP) determining HCC susceptibility in patients with different etiologies, i.e. EGF, IFNL3, MICA, and TLL1 in hepatitis C with or without active viral infection, and PNPLA3, TM6SF2, and MBOAT7 in metabolic liver disease.[16] Similarly, the AA genotype of SNP rs6726639 of MERTK, a gene involved in the polarization of macrophages in the tumor microenvironment and in activation of oncogenesis, showed to be strongly associated with HCC occurrence after DAA-induced SVR.[17] Conversely, the loss of function variant (rs72613567) in 17-beta-hydroxysteroid dehydrogenase 13 (HSD17B13) has been identified as protective of HCC occurrence in patients with alcoholic liver disease, but its role in hepatitis C should be further investigated, as HCV patients accounted for only 25% of the validation cohort on which these results were obtained.[18] Furthermore, a five-gene transcriptomic hepatic signature including angiopoietin-2, DLL4, NETO2, ESM1 and NR4A1, obtained by comparing tumor and non tumor liver tissues, was showed to be able to predict HCC growth and survival.[19] Interestingly, the study of epigenetic changes associated with HCV infection could be the key to explain the residual risk of HCC after SVR, especially in patients with advanced fibrosis. A recent analysis of liver tissues from patients with and without DAA-induced SVR identified specific genome-wide changes in H3K27ac, which correlated with changes in expression of mRNAs and proteins, that persisted after SVR and were significantly associated with HCC.[20] On the other hand, prior history of HCC recurrence and main nodule size resulted as independent predictors of HCC recurrence after DAA-induced SVR in patients with successfully treated early HCC.[21] Abnormal activation of neo-angiogenetic pathways and increased angiopoietin-2 are related to HCC recurrence and it has been suggested that DAA therapy could mediate an increase in VEGF levels in those with more severe liver fibrosis and portal hypertension.[22] Finally, the identification of circulating tumor products in the blood (liquid biopsy) is an attractive tool to improve precision medicine in patients with HCC and the feasibility of mutation detection by ultra-deep sequencing of circulating free DNA (cfDNA) in early HCC along with correlation of corresponding multiregional tissue samples was recently showed.[23]
In conclusion, an approach to HCC risk profiling based on the combination of clinical, genetic and epigenetic features could be useful to personalize clinical management according to the individual HCC risk, resulting in the improvement of the cost-effectiveness of surveillance programs, in the identification of possible targeted therapies and finally in the improvement of survival.

Aims of the project

- To assess the occurrence of HCC in a prospective cohort of patients with HCV chronic liver disease who achieved DAA-induced SVR.
- To assess the recurrence of HCC in a prospective cohort of patients with HCV cirrhosis who achieved DAA-induced SVR.after a successful treatment of early HCC.

- To assess clinical, genetic and epigenetic risk factors for HCC occurrence and for HCC recurrence and to develop an individual "risk profiling" to improve HCC surveillance program in post-SVR follow up.
- To compare clinical, genetic and epigenetic risk factors between patients who developed HCC occurrence and patients who developed HCC recurrence after DAA-induced SVR.

Materials and Methods

This project will be conducted in two cohorts of patients with HCV chronic liver disease who achieved SVR after DAA therapy, prospectively enrolled at three tertiary referral centers of Gastroenterology and Hepatology (Policlinico Universitario "P. Giaccone" of Palermo, Policlinico Universitario "G. Martino" of Messina, Ospedale Maggiore Policlinico of Milano). The first cohort will include patients without history of HCC before DAA-induced SVR; the second cohort will include patients who achieved DAA-induced SVR after successful treatment of early HCC with surgical resection or ablation.

For both cohorts, individually criteria for diagnosis, staging of liver disease, baseline virologic evaluation, DAA regimens, and treatment outcomes will be registered. US and esophagogastroscopy (EGS) will be performed before the start of DAAs. Cirrhosis will be diagnosed according to liver biopsy or the presence of oesophageal/gastric varices or liver stiffness ≥ 12 kPa using FibroScan. Functional class of cirrhosis will be attributed by Child-Pugh and MELD score. SVR is defined as quantitative HCV-RNA below the lower limit of quantification at least 12 weeks or more after the end of treatment. Hepatic decompensation is defined as the occurrence of portal hypertensive bleeding, hepatic encephalopathy, ascites or jaundice.

We will exclude patients: 1) with HBV-coinfection; 2) with previous liver transplant (LT), or on LT waiting list, 3) who withdrew from therapy or who were not evaluable for SVR; 4) who had not undergone ≥1 US after DAA start; 5) with treated HCC but without complete radiologic response (CRR) after resection or ablation.

Patients without history of HCC

Follow-up for patients without history of HCC will include physical examination and laboratory evaluation every 3 months, and abdominal US scan every 6 months. When a focal liver lesion is identified during US surveillance, HCC will be subsequently confirmed by computed tomography (CT) and/or magnetic resonance imaging (MRI) and/or biopsy, and it will be treated, whenever possible, according to EASL guidelines.[10,24] In patients who will develop HCC occurrence, number and size of HCC lesions, Barcelona Clinic Liver Cancer (BCLC) stage, AFP levels and treatment received will be recorded. Patients with chronic hepatitis C without cirrhosis will be evaluated three years after DAA treatment to confirm the absence of HCC.

Patients with successful treatment of early HCC

Follow-up for HCC patients who achieved complete radiologic response (CRR) (assessed by CT or MRI) after successful treatment of early HCC with surgical resection or ablation will include physical examination, laboratory evaluation, and abdominal US scan every 3 months, in addition to dynamic CT or MRI every 6 months. HCC recurrence will be diagnosed according EASL guidelines.[10,24] HCC recurrences will be treated, whenever possible, according to BCLC schedule and EASL guidelines. [10,24] During DAA treatments, all patients were followed up monthly for clinical and laboratory evaluation and US was performed at month 3 of DAA therapy, and at any time when considered by clinical judgement.

Biological Analyses

Genetic assessment

Genotyping for selected SNPs will be carried out from genomic DNA isolated from peripheral blood collected in EDTA (QIAamp DNA Blood Mini Kit) using the TaqMan SNP genotyping allelic discrimination method. Commercial or custom genotyping assays with allele-specific Taqman probes will be used. The genotyping call will be done with SDS software v.1.3.0 (STEP One Plus Real Time System, Applied Biosystems).

Gene expression: Real time PCR and western blot

Liver samples stored at-80°C will be distrupted/homogenizated using the TissueRuptor apparatus (Qiagen) immediately before nucleic acid extraction. DNA, RNA and proteins from biopsy will be extracted using the AllPrep DNA/RNA Micro Kit (Qiagen) according to manufacturer's instructions.

A quantity of 0.5 µg of RNA will be used for cDNA synthesis with the QuantiTect Reverse Transcription Kit (Qiagen). The levels of target and control mRNA in the samples will be determined using a real-time reverse transcription quantitative PCR (RT-qPCR) approach.The detection will be performed using SYBR Green and Quantitect Primer Assays (Qiagen), which provide a PCR reaction with high efficiency and specificity. Results will be expressed as N-fold difference in target gene expression relative to the rRNA 18S gene compared with the expression in HepG2 (or others) cell line used as calibrator. Data analysis will be performed using Sequence Detection Software Version 1.3-7500 (Applied Biosystems). Protein lysates will be subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and blotted over night onto a nitrocellulose membrane (ECL membrane, Amersham) using an SDS electroblotting system (BioRad, Munich, Germany) and blots will be probed with specific monoclonal antibody over night. Filters will be incubated with a secondary antibody and specific proteins will be visualized with the ECL detection system (Amersham, Germany). Results will be expressed as signal intensity respect to GAPDH as loading control.

Circulating-free DNA(cfDNA) analisys

Circulating DNA will be isolated from 5 and 1 mL of plasma and serum, respectively, using the QIAamp Circulating Nucleic Acid Kit (Qiagen). Ultra-deep DNA sequencing will be performed for all exons of a panel of selected genes using Small Genome Sequencing, Exome e Target Region Sequencing approach.

Epigenetic modifications

Methylated DNA Immunoprecipitation (MeDIP)-Quantitative PCR Analysis will be performed with MethylMiner Methylated DNA Enrichment kits (Invitrogen, Carlsbad, CA, United States) starting from1 µg of genomic DNA isolated from tissue samples. The methylation status of the CGIs located within the 5'-UTR/first exon region of selected genes will be determined by qPCR of DNA from immunoprecipitated and unbound DNA using selected primer sets. Reduced Representation Bisulfite Sequencing (RRBS) Analysis of Genome-Wide DNA Methylation will be performed through library preparation, next-generation sequencing, data analysis, and digital methylation quantitation. Formaldehyde cross-linking and the Chromatin Immunoprecipitation Assay (ChIP) assay, with primary antibody against trimethylated histone H3 lysine 4 (Abcam, Cambridge, MA, United States), will be performed using Magna ChIPTM A – Chromatin Immunoprecipitation kits (Millipore Corporation, Burlington, MA, United States). Purified DNA from immunoprecipitated and input DNA will be analyzed by qPCR with the same primers used in the MeDIP.

Outcomes

The primary outcome of interest in the cohort of patients without history of HCC before DAA-induced SVR is HCC occurrence. Time to HCC occurrence is defined as the time between the end of DAA treatment and the HCC occurrence or last follow-up visit.

The primary outcome of interest in the cohort of patients who achieved DAA-induced SVR after successful treatment of early HCC is HCC recurrence. Time to HCC recurrence is defined as the time between CRR and the HCC recurrence or last follow-up visit.

Secondary outcomes are overall survival and hepatic decompensation.

Statistical analysis

Univariate and multivariate Cox regression analyses will be used to identify variables associated with HCC occurrence and with HCC recurrence in the two cohorts. Variables with p values ≤ .10 in the univariate analyses will be included in the final multivariate model. For all analyses, $P \leq .05$ will be considered statistically significant. Kaplan-Meier curves will be used to estimate the time to HCC occurrence and to estimate the time to HCC recurrence. Log-rank tests will be used to assess differences. The discriminating ability of models to predict HCC occurrence and HCC recurrence according to multivariate analyses will be evaluated by receiver operator characteristic (ROC) curves at different time points.

3 - Bibliografia / References

1. International Agency for Research on Cancer, World Health Organization. Cancer today (https://gco .iarc .fr/ today/ home).
2. IARC. Fact sheets by Population-Globocan-IARC. http://globocan. iarc.fr/Pages/fact_sheets_population.aspx
3. World Health Organization. Projections of mortality and causes of death, 2016 to 2060 (http://www .who .int/ healthinfo/ global_burden_disease/ projections/ en/).
4. Lok AS, Seeff LB, Morgan TR, et al. Incidence of hepatocellular carcinoma and associated risk factors in hepatitis C-related advanced liver disease. Gastroenterology 2009; 136: 138-48.
5. Simonetti RG, Cammà C, Fiorello F et al. Hepatitis C virus infection as a risk factor for hepatocellular carcinoma in patients with cirrhosis. A case-control study. Ann Intern Med. 1992;116:97-102
6. Di Marco V, Calvaruso V, Ferraro D, et al. Effects of Eradicating Hepatitis C Virus Infection in Patients With Cirrhosis Differ With Stage of Portal Hypertension. Gastroenterology. 2016 Jul;151(1):130-139.e2.
7. Calvaruso V, Cabibbo G, Cacciola I, et al. on behalf of Rete Sicilia Selezione Terapia–HCV (RESIST-HCV). Incidence of Hepatocellular Carcinoma in Patients With HCV-Associated Cirrhosis Treated With Direct-Acting Antiviral Agents. Gastroenterology. 2018 Aug;155(2):411-421.e4.
8. Cabibbo G, Celsa C, Calvaruso V, et al. Direct-acting antivirals after successful treatment of early hepatocellular carcinoma improve survival in HCV-cirrhotic patients. J Hepatol. 2019 Apr 6. doi: 10.1016/j.jhep.2019.03.027.
9. Dyson J, Jaques B, Chattopadyhay D, et al. Hepatocellular cancer: the impact of obesity, type 2 diabetes and a multidisciplinary team. J Hepatol 2014; 60: 110–17.
10. EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma. European Association for the Study of the Liver. J Hepatol 2018;69:182–236.
11. Singal AG, El-Serag HB. Hepatocellular carcinoma from epidemiology to prevention: translating knowledge into practice. Clin Gastroenterol Hepatol 2015;13(12):2140–2151
12. Arase Y, Kobayashi M, Suzuki F, et al. Effect of type 2 diabetes on risk for malignancies includes hepatocellular carcinoma in chronic hepatitis C. Hepatology. 2013 Mar;57(3):964-73.
13. El-Serag HB, Kanwal F, Richardson P, Kramer J. Risk of hepatocellular carcinoma after sustained virological response in Veterans with hepatitis C virus infection. Hepatology. 2016 Jul;64(1):130-7.
14. Yu ML, Lin SM, Lee CM, et al. A simple noninvasive index for predicting long-term outcome of chronic hepatitis C after interferon-based therapy. Hepatology. 2006 Nov;44(5):1086-97.
15. Asahina, Y., Tsuchiya, K., Nishimura, T. et al. alpha-fetoprotein levels after interferon therapy and risk of hepatocarcinogenesis in chronic hepatitis C. Hepatology. 2013; 58: 1253–1262
16. Fujiwara N, Hoshida Y. Hepatocellular Carcinoma Risk Stratification by Genetic Profiling in Patients with Cirrhosis. Semin Liver Dis. 2019 May;39(2):153-162
17. Calvaruso V, Grimaudo S, Petta S, et al. AA genotype of the deSNP rs6726639 of gene MERTK (MER Tyrosine Kinase) is associated with development of hepatocellular carcinoma after hepatitis C virus clearance. Journal of Hepatology 70, E728-E7.
18. Yang J, Trépo E, Nahon P, et al. A 17-Beta-Hydroxysteroid Dehydrogenase 13 Variant Protects From Hepatocellular Carcinoma Development in Alcoholic Liver Disease. Hepatology. 2019 Mar 25. doi: 10.1002/hep.30623. [Epub ahead of print]
19. Villa E, Critelli R, Lei B, et al. Neoangiogenesis-related genes are hallmarks of fast-growing hepatocellular carcinomas and worst survival. Results from a prospective study. Gut 2016;65:861-869.
20. Hamdane N, Jühling F, Crouchet E, et al. HCV-Induced Epigenetic Changes Associated With Liver Cancer Risk Persist After Sustained Virologic Response. Gastroenterology. 2019 Jun;156(8):2313-2329.e7.
21. Cabibbo G, Petta S, Calvaruso V, et al. Is early recurrence of he-patocellular carcinoma in HCV cirrhotic patients affected by treat-ment with direct-acting antivirals? A prospective multicentre study. Aliment Pharmacol Ther. 2017;46:688-695.
22. Faillaci F, Marzi L, Critelli R et al. Liver Angiopoietin-2 Is a Key Predictor of De Novo or Recurrent Hepatocellular Cancer After Hepatitis C Virus Direct-Acting Antivirals. Hepatology. 2018 Sep;68(3):1010-1024.
23. Labgaa I, Villacorta-Martin C, D'Avola D, et al. A pilot study of ultra-deep targeted sequencing of plasma DNA identifies driver mutations in hepatocellular carcinoma. Oncogene. 2018 Jul;37(27):3740-3752.
24. EASL-EORTC clinical practice guidelines: management of hepatocellular carcinoma. European Association for the Study of the Liver; European Organisation For Research and Treatment of Cancer. J Hepatol. 2012;56:908-943.

PALERMO 09/07/2019