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GENETIC, VIROLOGICAL AND CLINICAL FACTORS ASSOCIATED WITH HEPATOCELLULAR CARCINOMA DEVELOPEMENT IN PATIENTS WITH CHRONIC HBV AND HCV INFECTION

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1. INTRODUCTION

In the last twenty years, antiviral therapy with nucleo(s)tide analogues (NA) has considerably changed the natural history of chronic liver disease in patients affected by chronic HBV infection. Before NA therapy the main cause of morbidity and mortality was decompensated liver disease (LD) followed by hepatocellular carcinoma (HCC) **(Fattovich 2003)**.

Therefore the antiviral therapy improved the overall free survival and LD and HCC free survival patients. So it reduced HCC risk from 30% to 80%. Many studies revealed that patients treated with third generation NA, which are entecavir and tenofovir, have an HCC annual risk development ranging from 0.5% to 1.4%. These patients have not cirrhosis and they are treatment naïve. This risk is increased instead in patients with liver cirrhosis and treatment-naive varying from 0.9% to 5.4. **(Papatheodoridis, Chan et al. 2015)**. These data confirm previous data about the first generation NA in which the HCC annual risk ranged from 1.5% **(Liaw, Sung et al. 2004**) to 2.5% **(Papatheodoridis, Manolakopoulos et al. 2011)**. Thus Antiviral therapy has changed the natural history of

liver disease through a chronic HBV infection modification. This modification involves HBeAg seroconversion and a level of HBV-DNA under the lower limit of detection. However, patients with chronic hepatitis and cirrhosis still have a residual risk of HCC development. For this reason it is necessary to identify risk factors and predictors of HCC that can not be modified by antiviral therapy but which may contribute to the development of HCC.

2. OBJECTIVE

The objective of this study is to evaluate in a population of patients, consecutively followed at Gastroenterology Unit, affected by chronic HBV infection and clinical or biopsy diagnosis of chronic hepatitis and/or liver cirrhosis:

- the liver complications incidence (liver decompensation, HCC and death)
- not editable factors by antiviral therapy but which are responsible for HCC development. These are:

a) **polimorphism SNPs of MerTK gene**. Recently a genome wide association study identifies MERTk as responsible for fibrosis worsening **(Patin, Kutalik et al. 2012)**. MerTK is a tyrosine kinase receptor of the TAM (Tyro3, Axl, MERTK) family, which is overexpressed or ectopically expressed in a wide variety of cancers. This receptor is expressed in macrophages particularly in M2 which are responsible for oncogenesis **(Sica, Invernizzi et al. 2014)**.

b) **HBsAg levels serum (qHBsAg).** A study conducted on not treated Asiatic patients HBeAg negative with genotype

B and HBV-DNA < 2.000 IU/mL, showed that qHBsAg levels serum > 1.000 IU/mL are an indipendent risk factor for HCC development. However it is not yet known the behavior of this indicator during antiviral therapy with NA **(Tseng, Liu et al. 2012)**.

3. PATIENTS AND METHODS

Patients. It was evaluated a cohort of patients infected by chronic HBV consecutively followed from 1996 to 2015 in Gastroenterology Unit of Policlinico Universitario P. Giaccone of Palermo. 248 of 443 patients with chronic HBV infection were evaluated. These patients had an histological and/or clinical diagnosis of HBV chronic hepatitis and liver cirrhosis. They were treated from the time of diagnosis, with antiviral therapy with nucleos(t)ide analogs, according to the International guidelines (EASL) (European Association For The Study Of The 2012) with lamivudine, adefovir dipivoxil, entecavir, tenofovir disoproxil, telbivudina, monotherapy or in association therapy in those patients with viral mutations. The treatment's objective was achieving virological suppression (HBV DNA <20 IU / ml). Patients affected by hepatocellulare carcinoma diagnosis, HCV and HIV coinfection at presentation were excluded from the study.

Methods. Chronic HBV infection was defined by presence of HBsAg positivity to at least six months and with HBV-DNA over the normal range > 20 UI/ml according to COBAS® TaqMan® HBV Test v2.0, an in vitro nucleic acid

amplification for quantification of hepatitis B virus DNA on the serum or plasma and with linear range between 20 IU/mL e 170.000.000 IU/mL. The virological (HBV-DNA, HBeAg, anti-HBe) and biochemical parameters (transaminases, bilirubin, Quick Time, complete blood count, creatinine) were assessed before treatment and during it every of 3- 4 months. In those patients in which it was found an increase of the levels of viremia at least 1 log10 during the treatment, HBV polymerase gene was sequenced to detect the mutation (spontaneous or induced by the therapy) able to identify the resistance to antivirals therapy. After extraction, the HBV-DNA genome was purified and subjected to PCR to amplify the Pol gene.

The product obtained was subjected to automated sequencing, and the nucleotide sequences analyzed in order to search for specific mutations. This method has a sensitivity of 15 IU / mL. It was evaluated also the presence the coinfection HCV and HDV. The virological evaluation included quantitative HCV-RNA by reverse transcription-PCR using Cobas Amplicor HCV Monitor Test, v 2.0; Roche, Basel, Switzerland, and HCV genotyping by INNO-LiPA HCV II assay; Innogenetics, Zwijndrecht, Belgium), IgG and IgM againts HDV and HDV-RNA.

In all patients HCC was surveilled through abdomen ultrasound and AFP dosage every 6 months. The HCC diagnosis and staging was performed on histological or radiological criteria, according to current EASL guidelines(**European Association For The Study Of The Liver et al. 2012)**. Since the virological suppression time patients was followed every 3-4 months keeping HBV-DNA under normal limit to the last evaluation: in patients who don't develop HCC is the last clinical evaluation avaiable; while in patients who develop HCC is the HCC diagnosis time. In patients with clinical and histological cirrhosis diagnosis and small esophageal varices, an upper gastrointestinal endoscopy was performed every 1-2 years. The patients with large esophageal varices (> F2) were treated with beta-blockers or elastic band ligation according to the guidelines of BAVENO (**de Franchis and Baveno 2015)**.

qHBsAg level serum quantification

HBsAg level serum was performed at the time of virological suppression and at the last clinical evaluation. This last evaluation in those patients affected by HCC coincides with the time of HCC development, while inthose patients without

HCC is the last evaluation available. The HBsAg was quantified by the ARCHITECT method (sensitivity of ≤ 0.05 IU / mL.), which is an immunoassay of microparticles coated with anti-HBs by chemioilluminescenz designed to detect HBsAg.

Polimorphism SNPs MerTK gene determination

The DNA will be extracted out of the column using the chromatography affinity principle via QIAamp DNA Mini and Blood Mini Kit (Qiagen) or QIAamp Circulating nucleic acid kit (Qiagen). The yield and integrity of the DNA is tested by measuring optical density with a spectrophotometer UV (260/280 nm ratio <2) and by electrophoresis on agarose gel. The genotyping study of polymorphism (rs4374383) is performed in real-time PCR assays for allelic discrimination with TaqMan probes containing allele-specific markers such as fluorochromes FAM or VIC, which allow the operator you to highlight the two variants in any single nucleic acid sequence. If this method is not designed to efficiently reveal allelic variants (regions too rich CG) it is applied to the Sanger method of direct sequencing

Statistical Analisys

All data were analyzed using the statistical package SPSS (version 20.0; SPSS, Chicago, IL, USA). Continuous variables were summarized as mean and standard deviation (S.D.), and categorical variables as frequency and percentage. The differences between media and median are evaluated with t Student, chi Square and Fisher test. Correlation between variables was evaluated by Spearman correlation. Significance testing was two sided and set to **<** 0.05. Logistic regression analysis was used to determine independent associations with the presence of liver disease at baseline. Cox regression analysis was used to evaluate the variables associated with HCC occurrence during the follow-up. HCC was defined as one or more nodule in the liver with particular contrast enhancement. The baseline variables included in the analysis to determine the association with the presence of HCC were age, gender, HBeAg status, HDV status, HBV-DNA log, oesophageal varices and MERk and the variables included in the Cox analysis to determine the association with the HCC were age, gender, HBeAg status, HDV status, OV and MERTk SNPs. The proportion of patients who experienced HCC and

the time of the event were evaluated by Kaplan-Meier curves and log-rank analysis. A comparison between MERTk value in patients who developed HCC and those who died during follow-up was performed. Area under the receiver operating characteristic (AUROC) analysis was used to establish the best cut off value of qHBsAg to predict the presence of HCC. Sensitivity (Se), specificity (Sp), positive and negative predictive values (PPV and NPV), positive likelihood ratio (+LR) and negative likelihood ratio (-LR) were calculated using cut-offs resulting in a diagnosis of HCC. Logistic regression analysis was used to determine independent associations with the qHBsAg at baseline.

4. RESULTS

Four hundred forty three patients with clinical or histological diagnosis of chronic hepatitis B or cirrhosis (244 chronic hepatitis and 170 cirrhosis) were included in the study between 1996 to 2015. Only two hundrend forty eight patients, of which 134 (54%) with chronic hepatitis, and 114 (46%) with chirrosis, were administrated third generation antiviral therapy with NA (41,9%), 31% TDV, 69% ENT. They maintained a constant viral suppression under detectable limit (HBV DNA <20 IU/ml).

There are not statistically significant differences between the two groups in terms of sex, therapeutic and virological factors. The only difference between the two groups was the age of patients. Indeed the patients with cirrhosis are older than patients with chronic hepatitis (**TABLE 1**).

Among 248 patients with virologic suppression 222 (89.5%) did not develop HCC, while the other 26 (10%) developed HCC (**TABLE 2**).

Kaplan-Meier curve-analisys demonstrate that patients with HBV liver cirrhosis have an increased risk of developing HCC than patients with chronic hepatitis (p <0.0001 by log

rank test) with 2.9% annual incidence versus 0.5% of patients with chronic hepatitis (**FIGURE 1**).

Also Kaplan-Meier curves-analisys show that patients with cirrhosis have a higher risk to develope liver decompensation (p = 0.0002 by log rank test) and death related to liver disease (p <0.0001 by log rank test) than patients with chronic hepatitis (**FIGURE 2 and 3**). There is not difference in terms of HBsAg seroconversion (**FIGURE 4**)

rs4374383 SNP of MerTK gene as a predictor of hepatocellular carcinoma

Cox-multivariate analysis demonstrate that the hepatocellular carcinoma related factors are cirrhosis (HR 4.17 (1.11 - 15.67) p 0.03), esophageal varices (HR 2.73 (1:14 to 6:53) p 0.02) and MerTK (HR 00:34 (0:13 - 0.93) p 0.03) (**TABLE 3**). AG or AA genotype of polimorphism rs4374383 SNP of MerTK gene represents an additional risk for the development of hepatocellular carcinoma as demonstrated by the Kaplan-Meier curve-analisys (**FIGURE 5**).

qHBsAg level serum as a predictor of hepatocellular carcinoma

The analysis of qHBsAg serum was conducted in 75 of 114 patients with HBV liver cirrhosis, which were HBeAg negative.These qHBsAg was evaluated at the time of virological suppression and at the of hepatocellular carcinoma development in patients who develop HCC (9 patients 22%) and at the last clinical assessment available, for patients without HCC in 66 patients (88%). The analysis showed that the only parameter that differentiates the two groups is given by the serum HBsAg during the development of hepatocellular carcinoma, in both univariate and multivariate analysis (AdjustedHR (95% CI) p 1.003 (1.000-1.005) 0.047) (**TABLE 4**). There is a strong correlation with serum levels at the time of virologic suppression ($r2 = 0.525$, $p = <0.001$) evident on linear regression. Among the patients, who develop HCC it was also demonstrated a notable increase of qHBsAg serum levels (**FIGURE 6**). The qHBsAg better cut-off obtained using reciving operated curve, ROC curve, is 3098.38 IU/ml with an AUROC 0.562, a sensitivity of 50%, specificity of 89%, a PPV 89% and NPV 51% and an LR + 4.64 and LR- 0.56. So using the qHBsAg with the cut-off of 3098.38 IU/ml, it is possible to identify

patients with higher risk of HCC as shown by the survival curves according to Kaplan-Maier (Log rank test p: 0.005) (**FIGURE 7**).

5. DISCUSSION

Preliminary data conducted in a population of patients with chronic HBV infection with constant virological (HBV DNA <20 IU/ml) and biochemical (ALT value persistently normal) suppression by nucleo(s)tide antiviral therapy, show that in patients with compensated liver cirrhosis there is a residual risk to develop hepatocellular carcinoma. This risk seems to be independent by virological factors directly editable from antiviral therapy. The qHBsAg is a good diagnostic test to predict the hepatocellular carcinoma development in patients with virologic suppression. The progressive increase associated with values over 3000 IU/ml can identify patients with a high risk for HCC development.

Polymorphism rs4374383 SNP in the gene MerTK and in particular the AA and AG genotypes confer an additional risk to develop HCC among patients with chronic HBV infection and particularly among those with liver cirrhosis.

These data on one hand identify the possible risk factors of HCC among patients with HBV, while on the other hand can help to identify patients at risk to improve clinical surveillance and ultrasound HCC. However there are still open issues about HCC in patients with chronic HBV infection which are the timing of the assessment and evaluation of qHBsAg and the biomolecular mechanisms responsible for HCC.

6. TABLES AND FIGURES

	Chronic Hepatitis (134 patients, 54%)	Cirrhosis (114 patients, 46%)
Age (media ± SD)	50,6 ± 13,4	56,6 ± 13,4
Sex (n.,%)		
• Male	101 (75,4)	93 (81,6)
• Famale	33 (24,6)	21 (18,4)
HBeAg status (n.,%)		
• positive	31 (23)	7 (6)
• negative	103 (77)	107 (94)
HBV-DNA log viremia (media ± SD)	5,9 ± 2,3	5,8 ± 2,3
Biopsy (n.,%)		
• Yes	106 (79)	58 (51)
• No	28 (21)	56 (49)
First Therapy (n.,%)		
• LAM	49 (36,6)	50 (43,9)
• ADV	15 (11,2)	5 (4,4)
• LAM+ADV	4 (3)	13 (11,4)
• TELB	4 (3)	4 (3,5)
• ENT	41 (30,6)	31 (27,2)
• TDV	21 (15,6)	11 (9,6)

TABLE 1. Baseline features according to stage disease in patients with chronic HBV infection under antiviral therapy.

	Chronic Hepatitis (134 patients, 54%)	Cirrhosis (114 patients, 46%)
Liver Decompensation		
• Absent	133 (99.3%)	100 (87.7%)
• Present	1 (0.7%)	14 (12.3%)
Hepatocellular Carcinoma		
• Absent	130 (97%)	92 (80.7%)
• Present	4 (3%)	22 (19.3%)
Death		
• Alive	129 (96.3%)	90 (78.9%)
• Dead	5 (3.7%)	24 (21.1%)
Causes of death		
• Liver Decompensation	0	4 (16.7%)
• Hepatocellular Carcinoma	0	13 (54.2%)
• No liver related	5 (100%)	7 (29.2%)
HBsAg Seroconversion		
• Absent	125 (93.3%)	110 (96.5%)
• Present	9 (6.7%)	4 (3.5%)

TABELLA 2. Clinical events according to stage disease during antiviral therapy.

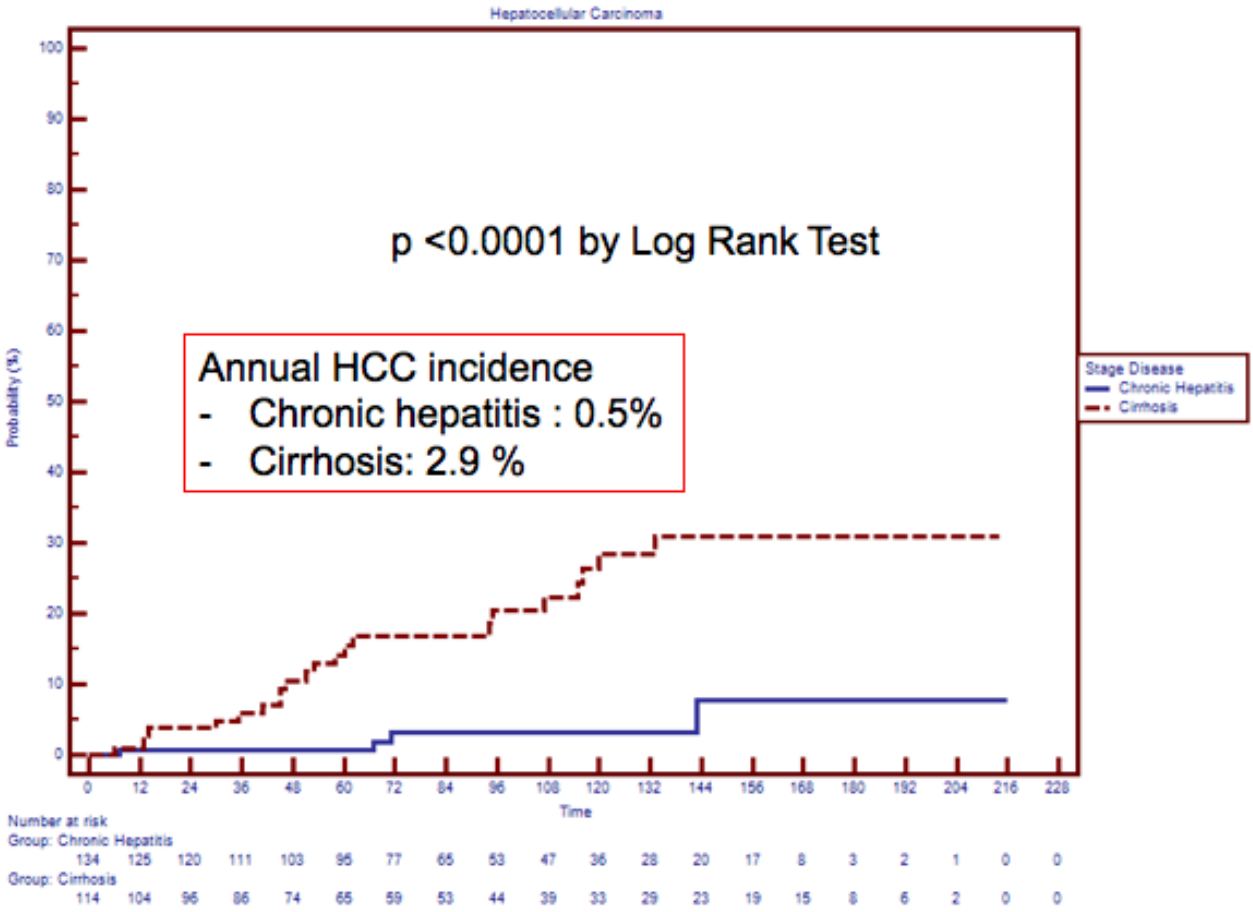

FIGURE 1. HCC developement risk according to stage disease.

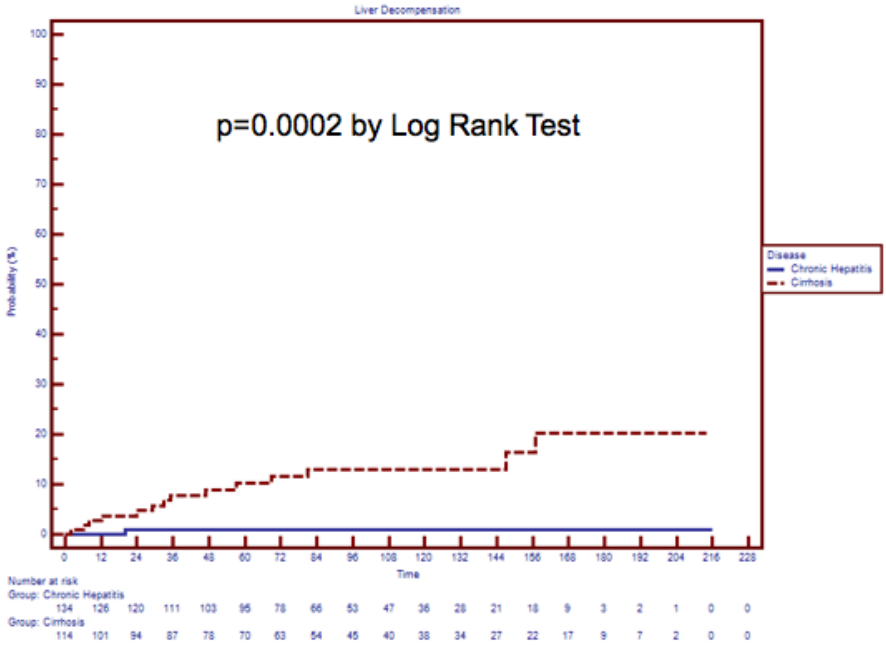

FIGURE 2. Liver decompensation risk according to stage disease during antiviral therapy

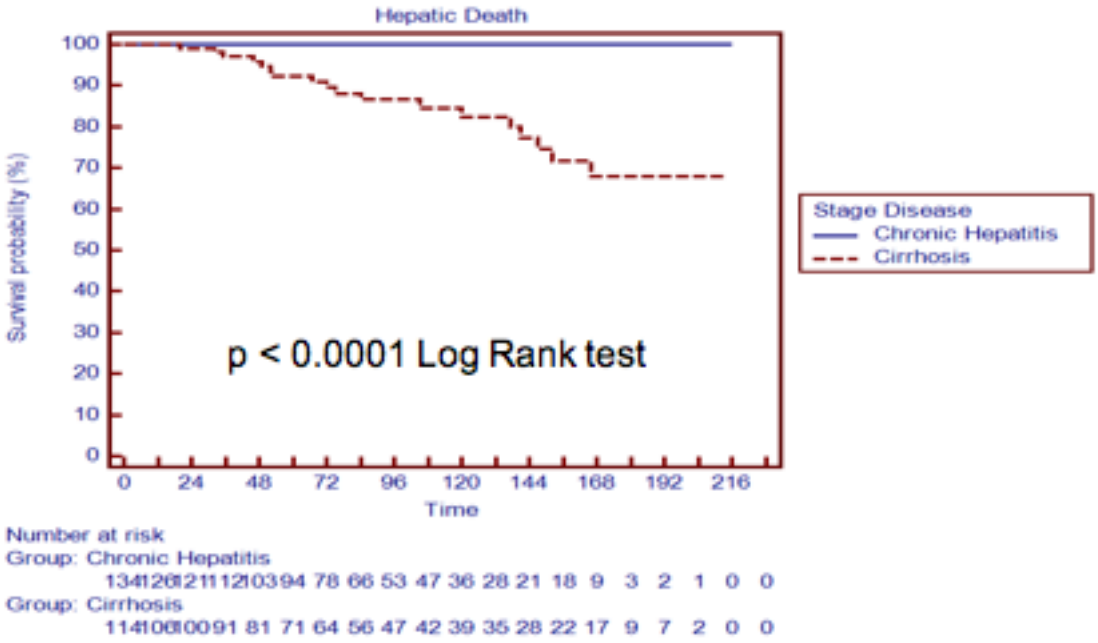

FIGURE 3. Survival analisys according to stage disease

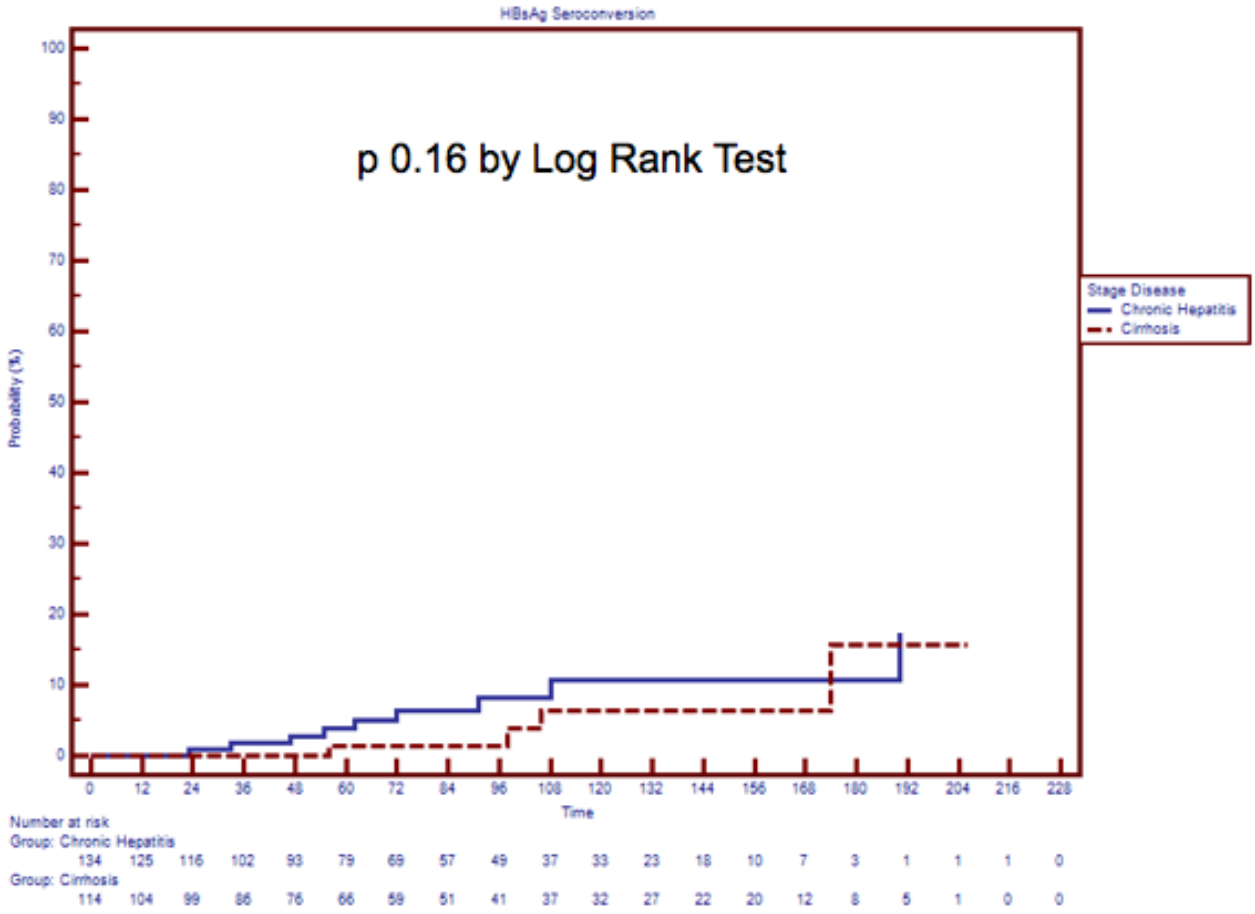

FIGURE 4. HBsAg seroconversion probability according to stage disease

	No HCC 222 pts (89.5%)	HCC 26 (10%)	p value	AdjustedHR (95%CI) p value
Age (years, mean, SD)	53 ± 12	60 ± 7	**0.004**	1.03 (0.98 – 1.09) 0.136
Gender • Male • Female	171 (77%) 51 (23)	23 (88.5) 3 (11.5)	0.183	
Stage disease • Chronic Hepatitis • Cirrhosis	130 (58.6) 92 (41.4)	4 (15.4) 22 (84.6)	**< 0.001**	**4.17 (1.11 – 15.67) 0.03**
HBeAg Status • Negative • Positive	192 (86.5) 30 (13.5)	23 (88.5) 3 (11.5)	0.780	
HDV Status • Negative • Positive	210 (94.6) 12 (5.4)	25 (96.2) 1 (3.8)	0.737	
Esophageal Varices • Absent • Present	178 (80.2) 44 (19.8)	12 (46.2) 14 (53.8)	**< 0.001**	**2.73 (1.14 – 6.53) 0.02**
MERTk SNPs • GA/AA • GG	113 (55.9) 89 (44.1)	19 (79.2) 2 (20.8)	**0.029**	**0.34 (0.13 – 0.93) 0.03**

TABLE 3. Risk factors for Hepatocellular Carcinoma (HCC) by Cox multivariate model

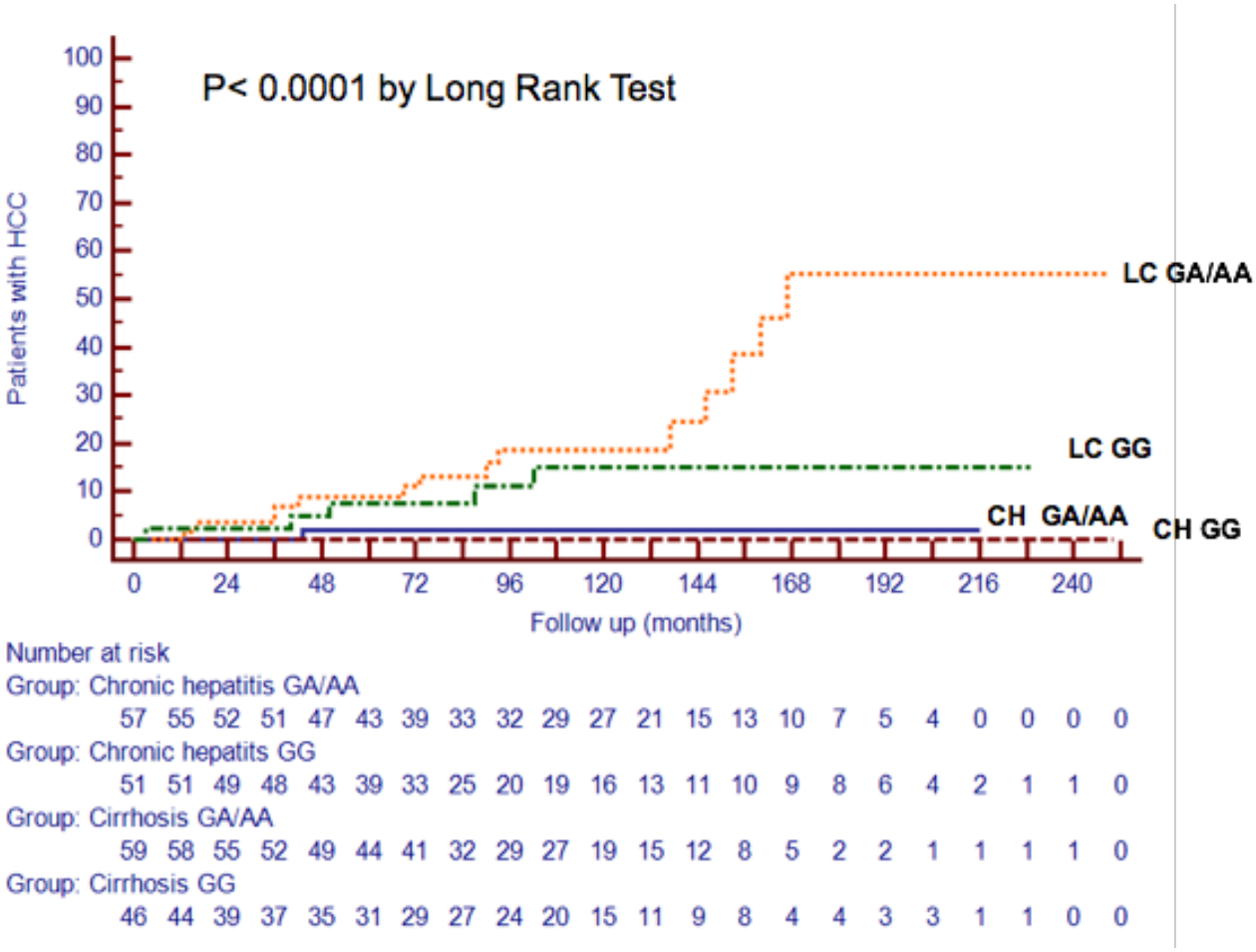

FIGURE 5. Hepatocellular Carcinoma risk according to stage disease and rs4374383 SNP of MerTK gene

Variables	Patients without HCC (n=66)	Patients with HCC (n=9)	p value	AdjustedHR (95%CI) p
Age (years, media ± SD)	51.3 ± 11.0	56.4 ± 12.6	0.188	
Sex (n%)	48 (82.8)	7 (100)	0.237	
Basal HBV-DNA (log, media ± SD)	5.1 ± 2.7	5.3 ± 3.7	0.798	
HBsAg to suppression time UI/ml (media ± SD)	2791.4 ± 2563.4	2709.2 ± 5130.2	0.942	
HBsAg to HCC time UI/ml (media ± SD)	1395.1 ± 1679.5	2687.7 ± 3086.7	**0.050**	**1,003 (1.000 – 1.005) 0.047**
Follow-up time (months, media ± SD)	121 ± 51.7	108 ± 55.8	0.467	
Time to suppression (months, media ± SD)	61.12 ± 32.4	55.9 ± 31.7	0.636	
Start Antiviral therapy (n%) LAM ADV TELB ENT TDV	40 (61.5) 7 (10.8) 4 (6.2) 11 (16.9) 3 (4.6)	5 (50) 1 (10) 0 (0) 3 (30) 1 (10)	0.721	
Last Antiviral therapy (n%) LAM ADV LAM+ADV ENT TDV ENT+TDV TELB +TDV SUSPENDED	2 (3.1) 1 (1.5) 0 (0) 17 (26.2) 39 (60) 2 (3.1) 2 (3.1) 2 (3.1)	0 (0) 0 (0) 1 (10) 3 (30) 5 (50) 1 (10) 0 (0) 0 (0)	0.266	
HBVpolimerase mutation (n%) (Wild-type) (LAM) (ADV) (LAM+ADV)	43 (66.2) 7 (10.8) 0 (0) 15 (23.1)	6 (60) 2 (20) 0 (0) 2 (20)	0.704	

TABLE 4. Risk factors associated with HCC developement in patients with HBV cirrhosis and HBsAg evaluation

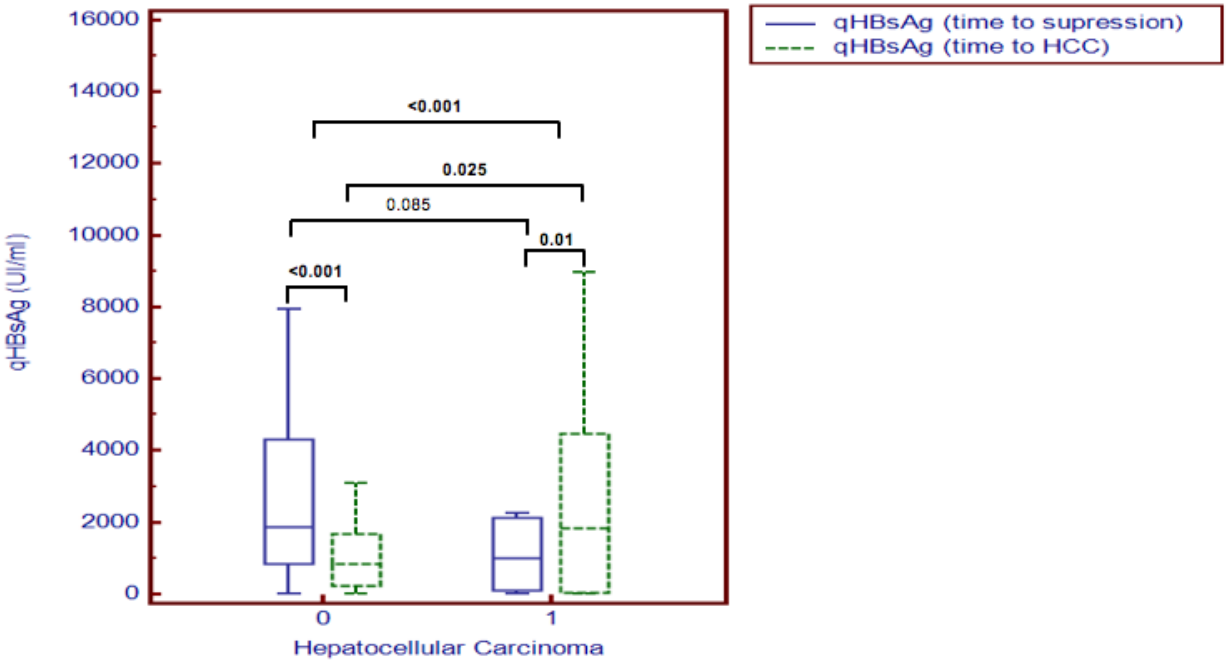

FIGURE 6. Box-plot dei livelli sierici di qHBsAg in relazione allo sviluppo di epatocarcinoma

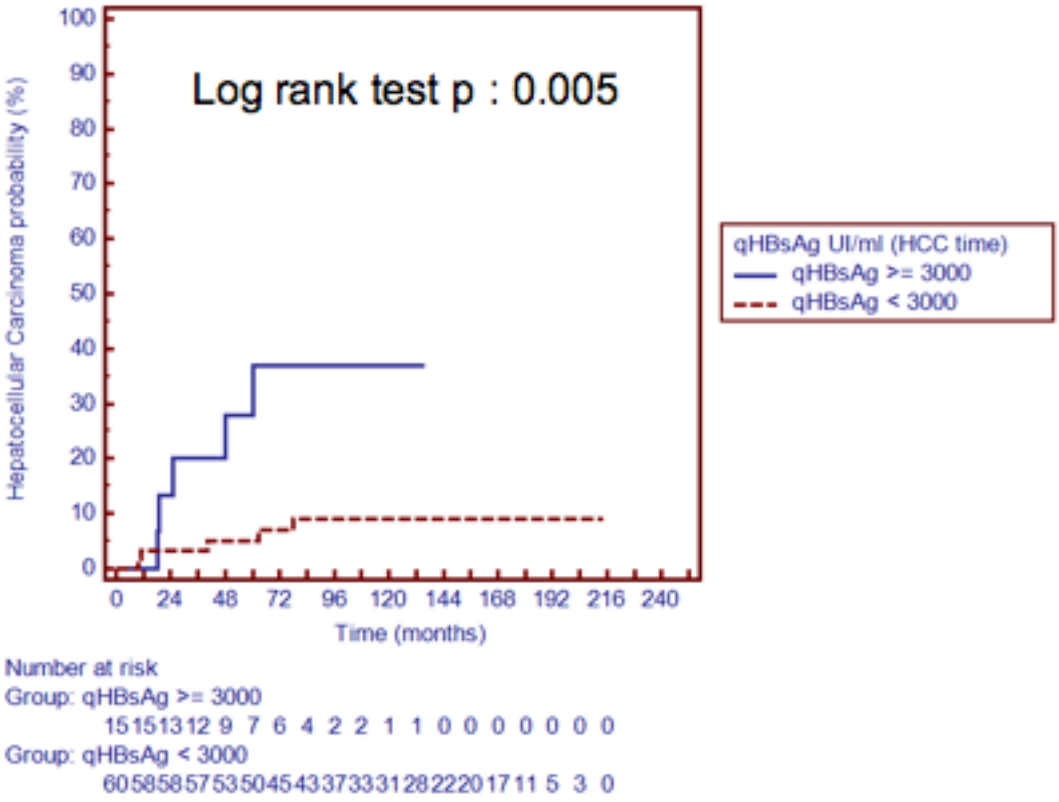

FIGURE 7. HCC risk according to qHBsAg serum levels with cut-off 3000 UI/ml

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