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**ANALYSIS OF THE EXPRESSION OF PRO-INFLAMMATORY CYTOKINES
AND VASCULAR ADHESION MOLECULES IN CIRRHOTIC PATIENTS
WITH OR WITHOUT HEPATOCARCINOMA**

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ABSTRACT

Background: Hepatocarcinoma (HCC) is the fifth most commonly diagnosed cancer and the second cause of cancer death in men worldwide, while in women represents the seventh most frequently diagnosed cancer and the sixth leading cause of cancer related death. HCC incidence is 10-20/100.000 per year and its mortality rate is 16/100.000 per year. 70-90% of patients are affected by liver cirrhosis and indeed this represents the greatest risk factor for HCC. Worldwide almost 54% of HCC is attributable to HBV infections, while 30% are caused by HCV and 15% by alcohol consumption. During progression from chronic liver disease to cirrhosis and then HCC, inflammation plays a pivotal role through changes in the hepatic microenvironment. Key factors are the cytokines IL-6 and Tumour Necrosis Factor α (TNF), and the adhesion proteins Vascular Adhesion Protein 1 (VAP-1), Intercellular Adhesion Molecule 1 (ICAM-1) and Vascular Cell Adhesion Molecule (VCAM-1).

Aims: The main aim of the study is to evaluate IL-6, TNF, VAP-1, ICAM-1 and VCAM-1 expression in serum samples of cirrhotic patients with and without HCC, thereby giving an interpretation of the role played by inflammation in the carcinogenetic process. Secondary endpoints are the identification of prognostic markers and future therapeutic targets.

Materials and Methods: Eighty consecutive patients with cirrhosis followed as outpatient controls between 2002 and 2012 were included. Twenty-two patients were affected by alcoholic cirrhosis, while fifty-eight presented liver cirrhosis and HCC. In the group of patients with HCC, forty-four patients underwent loco-regional therapies, whereas fourteen underwent surgery. Evaluation of cirrhosis was carried out by Child-Pugh, MELD and MELD-Na staging systems, ascites, encephalopathy, α -fetoprotein levels (α FP), tumour size and overall survival in HCC patients. A group of 20 healthy patients was used as control group.

The evaluation of VAP-1 serum levels was performed by *Enzyme Linked Immunosorbent Assay (ELISA - Bender MedSystems GmbH)* carried out on plasma samples after defrosting. The evaluation of the inflammatory cytokines (IL-6 and TNF- α) and of the adhesion soluble forms of cell adhesion proteins (ICAM-1 and VCAM-1) were performed by *Bio-Plex system (kit Bio-Plex Pro™ kit - Bio-Rad)* carried out on plasma samples after defrosting.

Numerical data were expressed as mean and standard deviation and compared with Student's t-test, while for categorical variables count and percentage in each category are reported and compared with Wilcoxon test. Cox regression and KaplanMeier method were performed for the survival study. Statistical significance was declared for P-value <0.05. The statistical analysis was performed with *STATA/SE 11.1 (StataCorpLP, College Station, Texas, USA)*.

Results: The analysis of the expression of pro-inflammatory proteins revealed that VAP-1 (p=0.001) and VCAM-1 (p=0.005) were significantly higher in cirrhotic patients without HCC compared to those with cancer. No statistical difference was assessed in levels of TNF, IL-6, ICAM-1, α -FP between the two groups. The group of patients treated with medical therapy showed a mean of 5-years survival of 42% and a median life expectancy of 38 months, while surgical patients showed a mean 5-years survival of 55% and a median life expectancy of 97 months. The univariate analysis demonstrated a statistical significance with a negative correlation with prognosis for ascites (p=0,012, HR 2,7, CI 95% 1,24-5,85) and tumor size (p=0,027, HR 1,01, CI 95% 1,24-5,85). No

statistical significance was found at univariate analysis for the other parameters (IL-6, TNF- α , VAP-1, ICAM-1, VCAM-1, Child-Pugh, MELD, MELD-Na, Encephalopathy, α FP). At multivariate analysis, tumor size and ascites were shown to be statistically significant, with a p respectively of 0.033 (HR 1,01, CI 95% 1,00083-1,02) and 0.02 (HR 2,54, CI 95% 1,159-5,57).

Conclusions: In this study VAP-1 and VCAM-1 were significantly higher in cirrhotic patients without HCC than in those with cancer. This could suggest a role of these molecules in the immune control. Even if in literature ICAM-1 is reported to have a higher expression in patients with cancer, in this study the analysis didn't show any differences between the groups. The hypothesis to be investigated is if the upregulation of ICAM-1 could lead the loss of immune control against the neoplasm. Moreover, also IL-6 and TNF were not statistically significant. Concerning to the prognostic impact, only the two clinical parameters ascites and tumor size were statistically significant with a negative correlation with prognosis, while none of the molecules have any correlation.

These preliminary results are not conclusive and further studies are needed to clarify the intriguing role of cytokines and vascular adhesion protein in carcinogenesis and their prognostic value.

The main limit of this paper is the lack of uniformity of the population studied. The different rate of staging subgroups in the various population may have affected the statistical analysis. A prospective case-control study is the next postdoc research topic. The first endpoint will be the homogenization of the population to be studied, thus allowing the evaluation of patients by stage (Child-Pough; Meld) and by etiology of cirrhosis (alcoholic, HBV, HCV), and furthermore making a comparison between the different categories and subgroups. Furthermore, as secondary endpoint, the study will focus on finding potential therapeutic targets and the subsequent therapeutic implication.

RIASSUNTO

Introduzione: L'epatocarcinoma (HCC) è la sesta neoplasia e la terza causa di morte per cancro a livello mondiale. La sua incidenza in Italia è di circa 10-20 casi/100.000 all'anno. Il principale fattore di rischio per HCC è la cirrosi epatica e infatti il 70-90% di pazienti con HCC ne è affetto. Nel 54% dei casi i pazienti con HCC hanno un'infezione da HBV, nel 31% da HCV ed nel 15% un'epatopatia da abuso alcolico. L'infiammazione, attraverso l'alterazione del microambiente epatico, gioca un ruolo fondamentale nel passaggio da epatopatia a cirrosi e infine a HCC. Fattori chiave dell'infiammazione indotta dall'alcool sono le citochine IL-6 e il Tumour Necrosis Factor α (TNF), e le proteine d'adesione Vascular Adhesion Protein 1 (VAP-1), Intercellular Adhesion Molecule 1 (ICAM-1) e Vascular Cell Adhesion Molecule (VCAM-1).

Scopo dello studio: Lo scopo dello studio è valutare l'espressione di IL-6, TNF, VAP-1, ICAM-1 e VCAM-1 nei pazienti affetti da cirrosi con e senza neoplasia per verificare il ruolo svolto dall'infiammazione nel processo di carcinogenesi. Ulteriore obiettivo è l'identificazione di eventuali fattori prognostici e di possibili bersagli terapeutici.

Materiali e metodi: Sono stati considerati 80 pazienti di cui 22 con cirrosi epatica, 44 con HCC insorto su cirrosi sottoposti a trattamenti loco-regionali e 14 con HCC insorto su cirrosi sottoposti a chirurgia. Tutti i pazienti avevano una cirrosi ad eziologia alcolica e sono stati trattati tra il 2002 e il 2012. Per ogni paziente è stato effettuato un prelievo di plasma precedente al trattamento o al momento della prima visita ambulatoriale. La cirrosi è stata stadiata mediante le classificazioni Child-Pugh, MELD e MELD-Na. Sono state inoltre valutati i seguenti parametri: ascite, encefalopatia, α -fetoproteina, dimensione del tumore, sopravvivenza. L'analisi dei livelli della forma solubile della VAP-1 è stata effettuata tramite test *ELISA*. IL-6, TNF, ICAM-1 e VCAM-1 sono state analizzate tramite il test *Bio-Plex Suspension Array*.

L'analisi statistica relativa a VAP-1, IL6, TNF- α , ICAM-1 and VCAM-1, α -FP è stata effettuata utilizzando il test parametrico T-test. Gli altri parametri sono stati studiati mediante il test non parametrico di Wilcoxon. La studio di sopravvivenza è statao effettuato utilizzando la regressione di Cox e il metodo di Kaplan-Meier method. La significatività statistica è stata indicata per $P < 0.05$. Per l'analisi è stato utilizzato il *software STATA/SE 11.1 (StataCorpLP, College Station, Texas, USA)*.

Risultati: Nell'analisi dell'espressione delle proteine proinfiammatorie nei pazienti cirrotici con e senza epatocarcinoma sono risultati significativi i valori di VAP-1 e VCAM-1. Questi sono risultati significativamente più alti nei pazienti cirrotici con un $p=0,001$ per VAP-1 e $p=0,005$ per VCAM-1. Le differenze di valori relative a IL6, ICAM-1, TNF e α FP non sono risultati statisticamente significative. Dopo un follow-up medio di 5 anni, la sopravvivenza mediana dei pazienti non operati è stata 38 mesi, mentre negli operati di 97 mesi. Solo le dimensioni del tumore e la presenza di ascite sono risultate significative all'analisi univariata, dimostrando entrambe un valore prognostico negativo (dimensioni del tumore $p=0,027$, HR 1,01, CI 95% 1,24-5,85; ascite $p=0,012$, HR 2,7, CI 95% 1,24-5,85). Allo stesso modo anche alla multivariata sono risultati significativi solo dimensioni del tumore ($p=0,033$ HR 1,01, CI 95% 1,00083-1,02) e ascite ($P=0,02$, HR 2,54, CI 95% 1,159-5,57).

Conclusioni: L'infiammazione promuove la tumorigenesi creando un microambiente infiammatorio e ossidativo che determina un danno cellulare e favorisce l'insorgenza di mutazioni genetiche. D'altro canto l'infiammazione contrasta l'insorgenza del tumore

tramite il processo dell'immunosorveglianza. IL-6, VAP-1, ICAM-1 e VCAM-1 giocano un possibile ruolo nel controllo immunitario della neoplasia, per cui nei pazienti con tumore dovrebbero presentare valori inferiori. In questo studio però solo VAP-1 e VCAM-1 hanno confermato questa ipotesi. TNF, IL6 e ICAM-1 non sono risultate significative nonostante il loro importante ruolo nel contesto infiammatorio e probabilmente anche nel processo della carcinogenesi, e pertanto il loro ruolo nell'epatocarcinoma rimane da chiarire mediante ulteriori studi. Inoltre queste molecole non sono risultate significative dal punto di vista prognostico, a differenza dei due fattori clinici, ascite e dimensione del tumore. Forse la gravità della patologia epatica della popolazione presa in studio ha mascherato il loro effetto.

Considerato quanto rilevato, i risultati preliminari non paiono conclusivi e pertanto sono necessari ulteriori studi per chiarire il ruolo delle citochine e delle proteine di adesione vascolare nella carcinogenesi. A riguardo nel prossimo lavoro l'obiettivo principale sarà quello di omogeneizzare il più possibile la popolazione in modo tale da poter suddividere la stessa per categorie di stadiazione e per eziologia, in modo da effettuare dei confronti fra i diversi gruppi con l'obiettivo di interpretare il ruolo di questi fattori nell'evoluzione dell'epatocarcinoma nei pazienti cirrotici.

BACKGROUND

Hepatocellular carcinoma (HCC) accounts 90% of all primitive liver neoplasms. It is the 6th commonest neoplasm and the 3rd cause of death for cancer worldwide. HCC is predominant among men and its mortality rate is 16/100.000 inhabitants per year in the world. Worldwide almost 54% of HCC is attributable to HBV infections, while 30% are caused by HCV and 15% by alcohol consumption.¹

Considered that 70-90% of patients are cirrhotic, almost all HCC arise in an environment of chronic inflammation and liver injury, suggesting that a proliferative and pro-inflammatory tissue state is a common feature of pre-neoplastic livers regardless of etiology.²

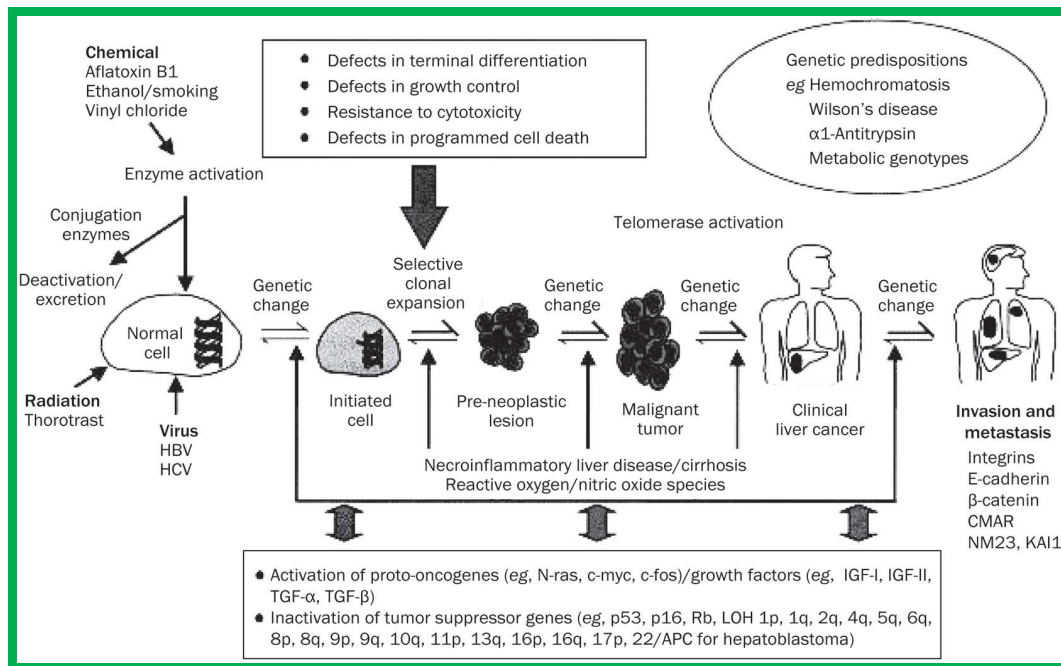


Fig. 1 The pathway from healthy cell to cancer³

During progression from chronic liver disease to cirrhosis and finally HCC, inflammation plays a pivotal role through changes in liver tissue leading to activation of several cellular pathways, which promote hepatocarcinogenesis by creating an inflammatory and oxidative microenvironment that causes cell damage and genetic mutations. Therefore, the inflammatory microenvironment plays a pivotal role in the beginning and progression of HCC.

The contact between immune cells surface proteins with hepatocytes, and the immune cells paracrine signals act on the preneoplastic environment, thus promoting cellular proliferation, genetic mutations and chromosomal instability. Once chronic inflammation has been established it follows a continuous production of cytokines and the recruitment of immune cells. So, if the inflammatory stimulus persists, the process is maintained and progresses. TNF and IL-6 are important cytokines in this process. Another important evidence is the activation of growth factors and their respective signalling pathways, which is a natural response to inflammation and liver damage with the aim of tissue repair and regeneration. Several growth factors are involved, among them Insulin Growth Factor (IGF), Hepatocyte Growth Factor (HGF), Wnt, Tumour Growth Factor Beta (TGF- β) and the signalling pathway of Epithelial Growth Factor Receptor (EGFR). In particular, pro-inflammatory cytokines stimulate EGFR ligands production, and in hepatitis and liver cirrhosis, as well as in HCC, an increased cyclooxygenase 2 (COX2) expression has been demonstrated. High prostaglandin E2 (PGE2) levels, produced by COX2 were found in malignant cells, thus suggesting a role in the initial phases of carcinogenesis.

FACTORS INVOLVED IN HCC CARCINOGENESIS

Hepatitis viruses^{2,4}

There is an increase of evidence regarding the ability of HBV and HCV viral proteins to cause oncogenic effects directly or to contribute to an increased risk of hepatocellular transformation in cooperation with the hyperproliferative response induced by chronic inflammation.

Hepatitis viruses cause chronic liver damage and therefore progression to fibrosis and cirrhosis. In any case, HCC may develop even in the absence of cirrhosis. The integration of HBV in the host genome causes deletions in DNA, translocations and mutations in various chromosomal positions. Several viral factors have been involved, including the genes HBx, pre-S2/S and HBV spliced protein. Among these, HBx is basic for the hepatocarcinogenesis process. It not only promotes the persistence of viral infection starting up the expression of the HBV genome and the replication, but also leads to genomic instability through the suppression of p53-regulated DNA repair

system. Unlike HBV, HCV is an RNA virus that is not able to perform a reverse transcription to DNA. Various HCV proteins such as the envelope and core proteins and non-structural proteins have been recognized as carcinogenic. It has also been described how these proteins are involved in the regulation of apoptosis and cell proliferation.

Alcohol⁵⁻¹²

Alcohol was recognized as a cause of chronic liver disease and cirrhosis. It may also affect the development of HCC by encouraging the initiation or promotion of the neoplasia through various processes:

- Formation of DNA adducts caused by acetaldehyde, which is produced by alcohol dehydrogenase (ADH) and CYP2E1 through the oxidative metabolism of alcohol
- Generation of reactive oxygen species (ROS) generated by the ethanol metabolism due to CYP2E1 and NADH oxidation

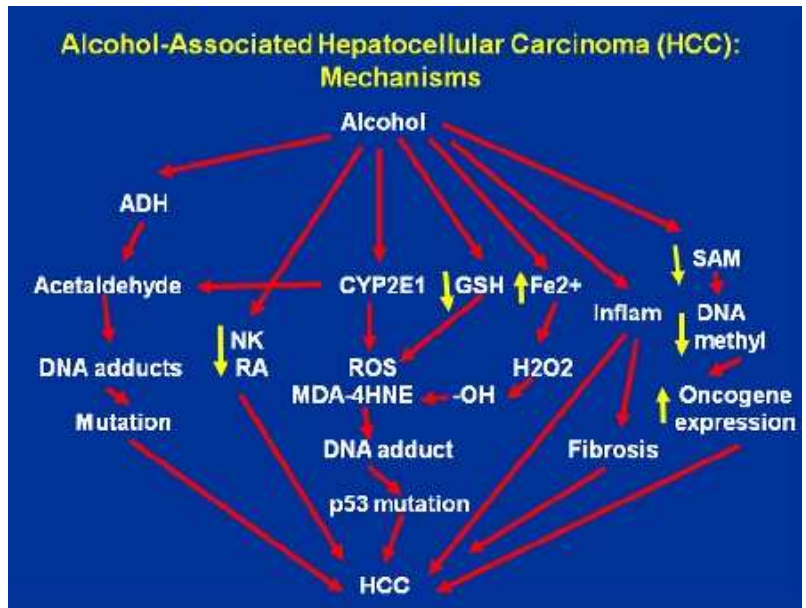


Fig. 2 Mechanisms of Alcohol-Associated Hepatocellular Carcinoma¹³

- Generation of ROS by the Fenton's reaction due to iron accumulation within Kupffer cells and hepatocytes
- Depletion of Glutathione enzymatic activity and liver concentrations
- Depletion of S-adenosylmethionine (SAM) resulting in DNA hypomethylation

- Depletion of hepatic retinoic acid concentrations and of retinoid signaling
- Induction of an inflammatory response thus facilitating chronic inflammation of and interruption of immunosurveillance
- Increase of intestinal permeability thus facilitating chronic inflammation
- Depletion of Natural killer (NK) cells levels and activity
- Activation of the HedgeHog pathway (HH).

Non-alcoholic steatohepatitis (NASH)

The main risk factors for the liver disease on metabolic basis are represented by visceral obesity, type 2 diabetes mellitus, hypertension and dyslipidemia, i.e. from that set of mutually correlated and influenced factors which is called metabolic syndrome. The course of the disease goes from steatosis to steatohepatitis up to fibrosis and hepatic cirrhosis. Steatohepatitis is characterized by the accumulation of fat vesicles in the cytoplasm of hepatocytes, like in steatosis, with in addition the presence of various degrees of lobular inflammation, swelling of hepatocytes, apoptosis, necrosis and fibrosis. Several mechanisms have been proposed to explain the process of carcinogenesis but, in any case, inflammation and generation of ROS play a key role. The latter perform the same function already seen in steatohepatitis of alcoholic nature.³ In addition the visceral fat produces a significant number of molecules, including TNF, IL-6, *monocyte chemoattractant protein* and leptin, secreted by the same adipocytes or macrophages there residing, leading to hepatic steatosis and oxidative stress through insulin resistance. A recent experimental study on murine models has shown how obesity facilitates the development of HCC by stimulating the production of cytokines that induce the development of the tumor, such as IL-6 and.¹⁴ As for insulin resistance, it is a typical characteristic of type-2 diabetes mellitus (DM2), and, indirectly, leads to an increased production of insulin in order to maintain adequate blood glucose levels.¹⁵ Insulin resistance and hyperinsulinemia, however, promote the rise of phosphorylation and activation of downstream signalling pathways which result in the inhibition of apoptosis and increased cell proliferation. Insulin resistance may also hasten hepatocarcinogenesis through stimulation of hepatic neovascularization. Hyperinsulinemia causes an increased activation of its own receptor, resulting in

phosphorylation of IRS-1 factor and activation of both the PTEN/ P12K/ Akt pathway and the MAP kinases pathway. PTEN is a tumor suppressor that reduces the stimulus towards Akt: its reduction encourages the development of cancer. Akt, on the contrary, promotes cell survival by inhibiting BAD protein, of the Bcl-2 proteins group, thus counteracting the phenomenon of apoptosis. Insulin causes the up-regulation of hepatic growth hormone (GH) receptors, which is the main stimulus for the production of IGF-1. The latter promotes cell proliferation both through the Akt pathway and through activation of MAPK and increased c-fos and c-jun proto-oncogene expression; it inhibits apoptosis and facilitates expression of epithelial growth factors. Hyperinsulinemia also reduces IGFBP1 and IGFBP2 hepatic synthesis, thus leading to an increased bioavailability of IGF-1. Leptin too is involved in the genesis of cancer: it is a predominant adipokine in adipose tissue, which acts at hypothalamic level reducing appetite and increasing metabolic consumption. Obesity in humans is often associated with high levels of leptin, suggesting that a mechanism of leptin resistance might be involved. Leptin secretion is stimulated by cytokines such as IL-1 and TNF, as well as by insulin, and increased levels are found both in chronic liver diseases and in HCC. In fact it contributes to hepatic fibrogenesis encouraging the expression of TGF- β , which in turn activates the stellate cells. As regards cancer leptin activates the JAK/STAT signal pathway which in turn facilitates the production of cyclin D1 and cell proliferation. Actually, the role played by leptin is far more complex, as evidenced by the fact that its expression is often increased in the tissues adjacent to the tumor and reduced in the tumor itself, suggesting the hypothesis that it might be involved not in cell proliferation but in the angiogenesis process. Finally adiponectin, a hormone produced only by adipose tissue, is markedly reduced both in obesity and in resulting steatosis, NASH, cirrhosis and HCC. It has an anti-inflammatory role, but cytokines (especially IL-6 and TNF) produced during the continuous chronic inflammatory condition of obesity and in the liver disease itself reduce its level. It also has an antiproliferative and pro-apoptotic effect through AMPK protein, which is able to stop the cell cycle through phosphorylation of other proteins, including for example mTOR.¹⁵⁻¹⁹

From chronic liver disease to cirrhosis and cancer

All the above mentioned factors lead to liver damage with inflammation and hepatocyte necrosis, facilitating the onset of a chronic liver disease. In this context, as a restorative response to injury, stellate cells acquire a myofibroblastic phenotype leading to an exaggerated deposit of extracellular matrix and then to hepatic fibrosis. This matrix deposit often results in reduced blood flow in some parts of the hepatic parenchyma. Fibrosis can progress to cirrhosis, during which irreversible architectural changes occur with nodule formation, while liver cells lose their normal arrangement. Cirrhosis is associated with cancer development but the molecular bases of this process are still poorly understood. However, evidence suggests that there is a sequence of transition from dysplasia to carcinoma in a similar way to what happens in other cancer types, such as in the colon. Probably the continuous process of necrosis and regeneration with an increased cell turnover makes liver cells more sensitive to the adverse effects of the various mutagenic agents. Genetic mutations occur of both genetic and epigenetic type, leading to the formation of dysplastic foci, nodules and eventually to HCC.^{3,20,21}

Genomic instability

The alteration of the genetic code is the first cellular event during carcinogenesis and is frequently found in many types of cancer. It is acknowledged that the integration of HBV DNA is a critical risk factor for genetic damage, but nowadays no specific genetic alteration has been found. Non-specific mutations in HCC generally involve β -catenin and p53. Furthermore erosion of telomeres, chromosomal segregation defects and oxidative DNA damage contribute to genomic instability.²

Oncogenes and signaling pathways

Hepatocarcinogenesis is a process closely related with the interruption of several signaling pathways involved in cellular survival, proliferation, differentiation, and apoptosis. These pathways are often intertwined and characterized by aberrant expression of protooncogenes or by the loss of tumor suppressor genes. p53 and retinoblastoma (RB) are two of the most frequently altered genes in cancer cells. Other members of the RB pathway too are often altered, for example D1/CDK4 cyclin, which

phosphorylates and inactivates RB, is overexpressed in 58% of HCC. P16, a negative regulator of RB, is absent in 34% of HCC. An aberration of Wnt signaling that leads to the development of cancer is often caused by an overactivation of β -catenin, whose mutation is estimated to be present in 20-40% of HCC. An activation of Akt with PTEN overexpression is present in 40% of cancers. Other key molecules are regulated by critical signaling pathways that are frequently altered in HCC, such as TGF- β /SMAD, Wnt/ β -catenin, IKK/NF- κ B. In addition to these ones, the Hedgehog and Notch signaling pathway, which plays a major role during the embryonic development, is often altered in HCC.^{2,22}

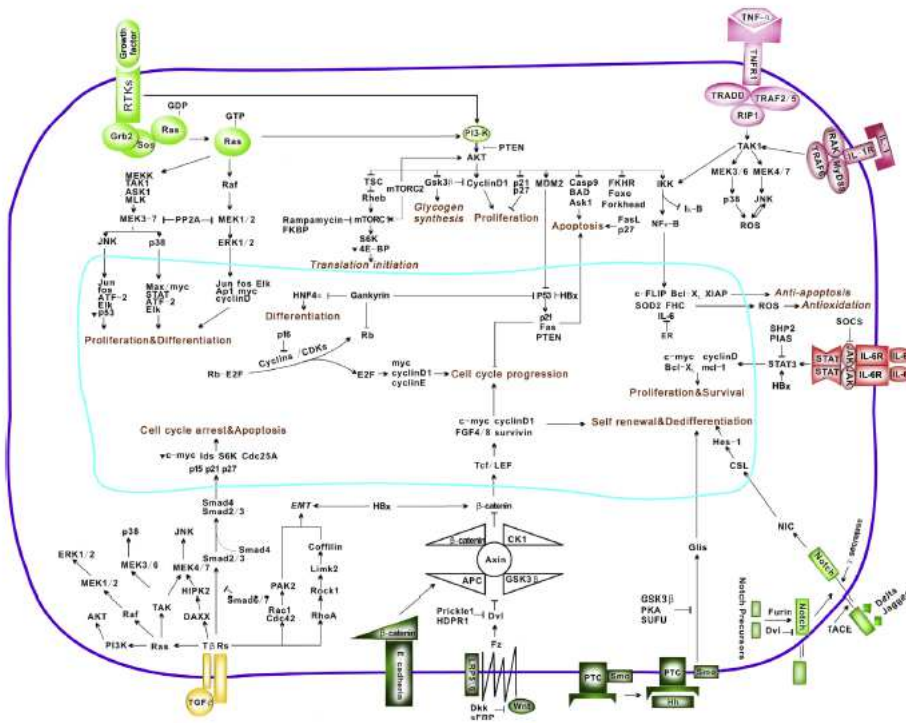


Fig. 3 Signaling pathway in Hepatocellular Carcinoma development²

Oxidative stress and HCC

Oxidative stress is caused by an excessive production of ROS and of reactive nitrogen species (RNS), and at the same time because of the inability to detoxify these products and repair the damage. The disposal of ROS and RNS is done mainly through glutathione molecules, thioredoxin and enzymatic systems such as superoxide dismutase, catalase and glutathione in the transcription of a large variety of cytokines and growth factors, which in turn can increase the levels of these reactive species. The

main sources of free radicals are represented by mitochondria and by P450 enzymes in hepatocytes, as well as by Kupffer cells and neutrophils present in the liver. They have a short half-life but they can interact with various classes of molecules such as proteins, membrane lipids, DNA and RNA. The caused damage may therefore lead the cell towards apoptosis or mutation of the genome. An important cellular defense mechanism is represented by various DNA repair systems, which are in turn, though, damaged by oxidative stress as well as by HBV infection.³

Cancer stem cells

Cancer arises from a small and defined subpopulation of cells defined as cancer stem cells (CSC). These cells have the self-renewal ability or can differentiate into mature cancer cells.²³ Scientific evidence supports the fact that CSCs are responsible for the hierarchical and heterogeneous organization of HCC. They are characterized by specific membrane markers such as EpCAM, CD13, CD24, OV-6, CD44 and ALDH. Many chemotherapy drugs are directed against mature cancer cells, having little or no effectiveness against CSCs; therefore it would be crucial to find some medicines suitable for this subpopulation. When CSCs migrate into the blood vessels they become circulating CSCs, playing a fundamental role in metastasization and formation of tumor-based portal thrombosis. A problem concerns the origin of these cells: adult hepatocytes still possess stem-like properties since they are capable of proliferation in response to a liver injury, being able to differentiate both towards hepatocytic lineage and, under particular circumstances, towards biliary lineage. It is possible that hepatocytes dedifferentiating may become CSCs and trigger the tumor. Another possible origin is from the hepatic progenitor cells, undifferentiated cells that normally reside quiescent in the bile ductules and in the canals of Hering, being able to differentiate towards both hepatocytic and cholangiocytic lineage. These cells when exposed to preneoplastic conditions, such as chronic inflammation, can turn into CSCs. Continuous exposure of TGF- β in vitro transforms these cells into CSCs. It is widely accepted that the development of HCC is caused by the propagation of these CSCs, although the molecular mechanism still remains unclear. Several studies have shown that there is a network of signals involving TGF- β , JAK/STAT3, Notch and PI3-K/Akt/mTOR.^{2,24}

Hepatic progenitor cells are small cells with a large nucleus and a thin rime of cytoplasm around; in animals they are also called oval cells. Recently it has been shown that in a cirrhotic liver cells become senescent due to a shortening of telomeres. Some senescent cells acquire additional mutations and a subsequent gain in replicative capacity, making it likely that at least a part of HCC arise from a hepatic progenitor cell.

Tumor microenvironment

Several studies have shown how the tumor microenvironment plays a pivotal role in tumorigenesis and neoplastic progression. Cancerous cells are not as autonomous as it was thought once: they are dependent on angiogenesis, inflammatory cells and fibroblasts. Obviously in the cirrhotic liver there are plenty of fibroblasts, and there is evidence that some of them are cancer-associated fibroblasts (CAF). In HCC the progression of malignant hepatocytes often depends on the TGF- β provided by stromal cells (macrophages, fibroblasts etc.). This molecule is one of the factors that may induce a mesenchymal-epithelial transition (EMT). For different types of cancer it has been noticed that an EMT is associated with a worse prognosis, such as for example in esophageal squamous cell carcinoma, in stomach, bladder cancer, in non-small cell lung cancer as well as in pancreatic ductal adenocarcinoma. The characteristics of EMT are a change in gene expression with overexpression of twist, snail, VE-cadherin, vimentin and a down-regulation of E-cadherin, HNF4 α hepatocyte transcription factor and changes in the cytoskeleton. In EMT there is a destabilization of anchoring junctions and the cell develops enhanced invasive properties. In animal models, it has been noticed how EMT promotes neoplastic invasion and metastasization. Both HBV and HCV, in addition to a hypoxic condition, promote EMT.^{3,25-29}

Hypoxia and tumor progression

Hypoxia is involved in the angiogenesis process through the HIF- α 1 pathway which stimulates the expression of pro-angiogenic factors, as well as induces EMT. Furthermore, chronic exposure to an hypoxic environment is likely to lead to an adaptation of the genetic expression profile of cancer cells, giving them a more aggressive behavior.³

INFLAMMATION AND CARCINOGENESIS

Epidemiological studies have established a relationship between chronic inflammatory diseases and cancers, and it is well known that a persistent inflammatory condition increases the risk of cancer and facilitates carcinogenesis. The presence of an inflammatory microenvironment plays an essential role in HCC initiation and progression. The infiltration of inflammatory cells can be caused by necrotic or apoptotic hepatocytes. Various cytokines and chemokines, secreted by immune cells and the direct contact between the surface proteins of immune cells and hepatocytes, remodel the preneoplastic microenvironment, promoting genetic mutations and cell proliferation. TNF and IL-6 are the main cytokines in this process.²

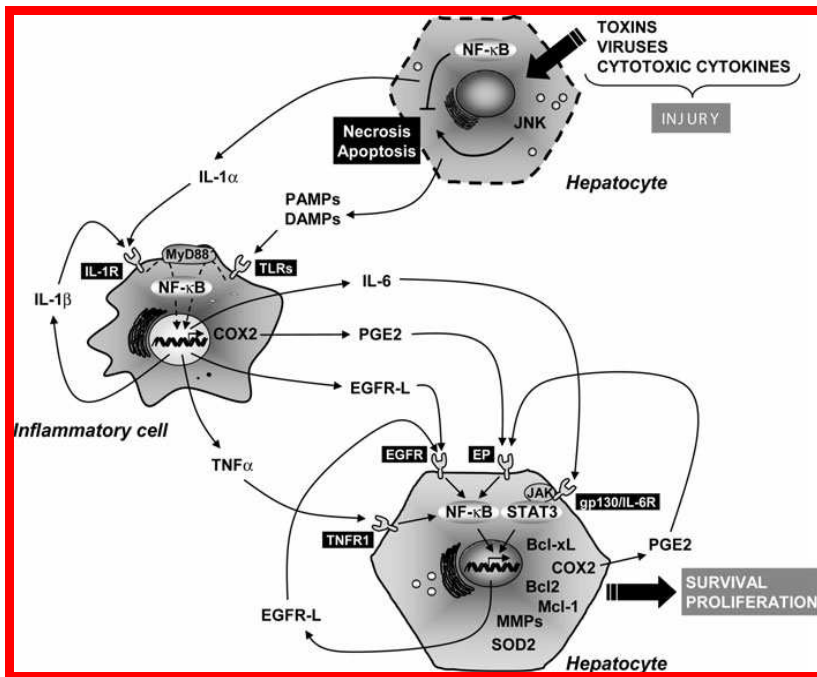


Fig. 4 Vie molecolari che collegano l'infiammazione allo sviluppo di HCC³⁰

Another common finding in chronic inflammatory liver diseases and in HCC is the activation of growth factors and their respective signaling pathways. This is a natural response to inflammation and damage of liver tissue with the aim of a tissue regeneration and repair. In any case, when chronically stimulated or dysregulated by molecular or functional changes, these growth factors may contribute to the neoplastic transformation and to the maintenance of the transformed phenotype of HCC cells.

There are different growth factors involved, including insulin-like growth factor (IGF), HGF, Wnt, TGF- β and the EGFR signaling system. In particular in murine models it has been noticed that HGF is involved in carcinogenesis through autocrine activation of the HGF-Met pathway. Furthermore, overexpression of EGFR has been described in HCC samples, and a correlation has been identified between high levels of EGFR and adverse prognosis. Similarly also EGFR ligands (TGF- α , HB-EGF, BTC and AR) have been found in a high percentage of HCC. On the contrary the specific deletion of EGFR in the liver parenchyma of murine models results in a weakening of hepatic proliferation in the early stage of regeneration, and the specific deletion of its ligands leads to a reduction of cell proliferation. The administration of gefitinib, an inhibitor of EGFR molecule, prevented chemically induced HCC in mice. All this evidence support the involvement of a dysregulation of EGFR in the multistep process leading to carcinogenesis. Important informations have also been obtained on its interaction with the classic signaling pathways involved in inflammation. These interactions can be observed at different levels from the mechanisms that regulate expression and availability of EGFR ligands, including their cellular origin, to their effect on target cells. Expression of EGFR ligands is generally increased in acute and chronic inflammation. Within the liver parenchyma these factors can be produced not only by hepatocytes and by cells that produce extracellular matrix, but also by inflammatory cells such as Kupffer cells and resident macrophages. This has been demonstrated for AR, HB-EGF and TGF- α .³⁰⁻³⁵ Moreover the platelet derived growth factor (PDGF), produced by the activated Kupffer cells, can facilitate the production of AR in cells that produce extracellular matrix. Even expression of HB-EGF can be induced in non-parenchymal liver cells through stimulation of LPS. Furthermore liver cells may express different EGFR ligands when stimulated with pro-inflammatory cytokines such as for example TGF α under stimulus of TNF and AR for IL-1 β . In addition TGF- β , another growth factor produced by non-parenchymal liver cells during inflammation, can facilitate the expression of HB-EGF and TGF- α . Proinflammatory cytokines, besides stimulating the expression of these ligands, can also induce their release on cell surface thanks to the mediation of ADAM family metalloproteinases. Thus, it is not surprising that the expression of ADAM17 is overexpressed in chronic liver diseases and HCC.

The EGFR signaling system has relationship with many other pathways involved in inflammation, as that one mediated by COX2 which results in prostaglandin production. The importance of this pathway has already been demonstrated in colon cancer, but it is also involved in hepatocarcinogenesis. Increased expression of COX2 has been found in hepatitis and liver cirrhosis, as well as in both experimental and human HCC. High levels of PGE₂, produced by COX2, can be found in malignant cells and they are thought to contribute to the initial stages of carcinogenesis, including cancer cell proliferation, cell survival, invasiveness, angiogenesis, and metastasizing ability. Data are now available also about how, in both experimental and human HCC cell lines, COX2 inhibitors have the ability to inhibit the cell growth. The mechanisms have not yet been understood clearly, even if there seems to exist a strong correlation between EGFR signaling pathways and COX2. Probably the activation of EGFR up-regulates the expression of COX2, which in turn, through the production of PGE₂, activates EGFR. PGE₂ is also able to facilitate the expression of EGFR ligands such as AR. COX2-derived prostanoids could be a key signal in the activation of EGFR from the early stage of liver inflammation to cancer.^{30,36-38}

IL-6

IL-6 is a pleiotropic cytokine, expressed in many inflammatory cells in response to different types of stimuli and cellular processes, including cell proliferation and differentiation, and plays a central role in the acute phase response and in the control of balance between proinflammatory and anti-inflammatory pathways. High levels of IL-6 have been found in several inflammatory and neoplastic conditions, and particularly in liver diseases as chronic viral hepatitis, alcohol-related liver disease, liver cirrhosis and HCC.^{39,40}

Chronic inflammation plays a central role in cancer initiation and promotion. The effect of the immune system in the inflammatory process that leads to carcinogenesis is the stimulation of tumor growth. In the case of chronic inflammation, the balance between Th1 and Th2 cytokines in the adaptive immune response is often altered in cancer patients, and increased levels of Th2 cytokines can enable an immune escape of cancer cells. In fact, high levels of Th2 cytokines such as IL-4, IL-6 and IL-10 are associated

with adverse prognosis. Furthermore, IL-6 is a negative independent prognostic variable factor in patients with HCC. IL-6 levels also have a positive correlation with tumor size and stage of HCC, as well as with tumor aggressiveness. IL-6 is indeed considered as a Th2 cytokine, although actually it takes part in both Th1 and Th2 processes. This all supports the hypothesis that in the tumor a shift from Th1 to Th2 immune responses may suppresses the host antitumoral immune response, promoting the neoplastic growth.

Several studies have suggested various biological mechanisms for the hepatocarcinogenic action of IL-6. Liver cirrhosis is associated with increased levels of mRNA expression of IL-6, with increased values according to Child-Pugh class, and IL-6 gene expression is up-regulated by the hepatitis B virus x protein (HBx), leading to oncogenic transformation.⁴¹⁻⁴³ Obesity and diabetes mellitus are recognized risk factors for HCC: it has been shown that visceral fat leads to an increased expression of cytokines, such as for example TNF and IL-6.¹⁴ It was also demonstrated how the HCC cells can produce IL-6, by stimulating the tumor growth through an autocrine mechanism.⁴³ IL-6, produced by inflammatory and stromal cells within the tumor microenvironment, binds to the gp80 (IL-6 receptor)/gp130 complex, leading to the activation of a constitutive Janus kinase (Jak) and then to STAT3 phosphorylation, which finally can activate the expression of oncogenes that mediate the proliferation and inhibit apoptosis.^{14,44} Finally it has been noticed how some polymorphisms of this cytokine are associated with an increased risk of both chronic liver disease and HCC, in particular 174 G/C polymorphism, which involves the IL-6 gene regulatory region, leading to an overexpression of the cytokine itself. It has been noticed that in this group the risk of HCC is increased compared with the one with the normal variant of the IL-6 gene.⁴⁰

Tumour necrosis factor α (TNF)

Among the factors secreted by inflammatory cells associated with tumors, TNF has been identified as one of the main molecule that regulate inflammation and it is considered a potential mediator in cancers related to inflammation.⁴⁵ Therefore TNF is a powerful cytokine that regulates cell proliferation, survival, migration and angiogenesis.^{46,47}

To promote all these changes it acts through various pathways. First, through TNFR1 factor it activates the NF- κ B key inflammatory transcription factor. It plays a very complex role in cancer development, in fact its inactivation leads to a greater number of tumors in mice treated with the carcinogen DEN. Conversely, inhibition of NF- κ B in parenchymal cells in the later stages of cancer results in a reduction of tumor progression. These apparently conflicting data actually must be evaluated depending on the time period of inhibition of NF- κ B and the various cellular targets that are involved. It has been shown how the loss of NF- κ B in hepatocytes elicits carcinogenesis induced by chemical compounds through the activation of c-jun N-terminal kinase 1 (JNK1). In addition, the deletion of JNK1 causes a reduced hepatocellular damage and a block in the development of HCC, confirming *in vivo* the cytotoxic consequences of a prolonged activation of JNK1. These observations, when considered all together, show the essential role of NF- κ B and of its dialogue with JNK1 in protecting hepatocytes. Therefore the preservation of hepatocyte survival may indirectly reduce tumorigenesis by attenuating the compensatory proliferative response during cirrhosis, which allows hepatocytes, even with mutations, to enter the cell cycle.^{48,49}

TNF also activates other molecules. One of these is Forkhead box M1 (FoxM1), a ubiquitous transcription factor that, by affecting different target genes, acts as a key regulator of the cell cycle both in the transition from G1 to S phase, and in the progression to the mitosis phase. Several studies have reported that FoxM1 is overexpressed in HCC cells and is an indicator of adverse prognosis. In addition to TNF, it is overexpressed through the RAS pathway and also under hypoxic conditions, in fact hypoxia-inducible factor (HIF-1) is a critical mediator in the induction of FoxM1 by TNF. The coexpression of FoxM1 and HIF-1 in HCC cells is an indicator of adverse prognosis. Finally another way in which TNF can facilitate the process of carcinogenesis is represented by its own pro-inflammatory action, which increases the generation of ROS and subsequent cellular damage. The same production of ROS, moreover, is able to stabilize the HIF-1 factor, and then facilitate the proliferative pathway mediated by HIF-1 and FoxM1.^{45,50,51} Furthermore, TNF stimulates the production of TNF-induced protein 8-like 2 (TNFAIP8L2), which acts as negative regulator of innate and adaptive immunity. It also facilitates cell apoptosis, induced by caspase 8 and Fas. It has been

noticed that this protein is able to inhibit the metastasization process of HCC, whereas its lack facilitates the spread of the tumor. Its action is mainly due to the ability to suppress the polymerization of F-actin and the expression both of metalloproteinase-9 and of the urokinase-type plasminogen activator (uPA), which are essential for the migration and cell proliferation leading to the metastatic process.⁵² Finally, as for IL-6, the presence of polymorphisms that add an increased risk of HCC has been detected. In particular the 308 G > A variant has been evaluated in several studies, and a meta-analysis carried out on 20 studies with 2763 patients with HCC and 4152 controls has shown an OR of 1.84 for this polymorphism.⁵³

Vascular Adhesion Protein 1 (VAP-1)

VAP-1 is a human endothelial sialoglycoprotein whose cell expression is induced in case of inflammatory conditions. It participates in the lymphocyte recirculation mediating their link with endothelial cells.⁵⁴ VAP-1 forms a homodimer of 180 kDa, expressed on vascular endothelium. In vitro it has been noticed how essential it is for the mechanism of leukocyte rolling, for the firm adhesion and the migration during the extravasation process.⁵⁵ In addition to these adhesion abilities it also has enzymatic properties. Indeed it is also referred as semicarbazide-sensitive amine oxidase (SSAO), or aminoxidase containing copper (AOC3), which catalyzes the oxidative deamination of primary amines. This is not a matter of secondary importance, since its catalytic ability is as fundamental as the adhesive ability.⁵⁶ It is expressed early in utero and is functionally active long before birth. It is likely to contribute to oxidasic activities, besides being of crucial importance in lymphocyte traffic during the ontogenetic process.^{55,57} VAP-1 is a fundamental protein involved in several inflammatory processes, in fact it promotes lymphocyte homing on endothelial cells at the level of the peripheral lymph nodes and of inflammation sites. It is constitutively expressed on endothelial cells of the human liver but not very present in the blood vessels of non-lymphoid organs in non-inflammatory conditions.⁵⁸ In these cases it is stored in intracellular granules and then brought to the surface if needed.⁵⁹ It is up-regulated in the high endothelial venules of the peripheral lymph nodes in case of inflammation. Its expression is increased in experimental models of arthritis, in the inflamed lung

compared to the normal lung and in endotoxin-induced uveitis.^{58,60} It was also pointed to as a factor involved in the rejection of liver transplant.⁶¹ The enzymatic activity of VAP-1 is also responsible for the formation of free radicals such as hydrogen peroxide and ammonium that lead to a cytotoxic damage of the endothelial cells. In addition, hydrogen peroxide is an important signaling molecule that leads to an increased expression of CD62P and VCAM-1 adhesive proteins (vide infra), as well as to chemokine and metalloproteinase receptors. In this way the ongoing inflammatory process is enhanced. VAP-1 is also involved in inflammatory processes relating to cardiovascular diseases, diabetes, obesity and hypertension. All this makes this molecule a possible target for inhibitor drugs.⁶⁰

Another important correlation exists between VAP-1 and diabetes: as previously stated VAP-1 levels are increased in diabetic subjects and it accounts for the pathogenesis of some of its complications such as for example retinopathy. In the retinal vessels VAP-1, along with other adhesion proteins such as ICAM-1 and VCAM-1, facilitates the leukocyte adhesion, thereby contributing to the genesis of diabetic retinopathy.⁶²

Altered expression of VAP-1 is present in several types of cancer: in stomach cancer low levels of sVAP-1 (soluble form of VAP-1) are associated with adverse prognosis.⁶³ In the same way lower levels are present in colon cancer and these are associated with adverse prognosis besides being predictive of lymphatic and hepatic metastases.⁶⁴ These results are explained by the fact that the tumor infiltrating lymphocytes (TILs) act against the growth of the tumor.^{63,64} On the contrary in melanoma the intratumoral vessels have been shown to possess low levels of VAP-1 while the peritumoral vessels overexpress VAP-1. This difference is probably due to the transport of different types of molecules, some of which are probably involved in angiogenesis with respect to the peritumoral vessels.⁶⁵

In the liver the hepatic endothelial cells form a specialised endothelium that supports lymphocyte adhesion and recruitment in a low flow optimal environment. The hepatic endothelium has a different phenotype than other vascular beds. In vivo the hepatic endothelium expresses low levels of CD31, which is among the most represented adhesion factors in other tissues. VAP-1 in the hepatic endothelium promotes lymphocyte adhesion in laminar shear stress conditions. It is a mediator of the

transendothelial lymphocyte migration but its ability to facilitate cell adhesion and migration is blocked by specific inhibitors of its enzymatic activity. In some studies increased levels of VAP-1 in patients with HCC have been identified. These findings suggest that increased levels of VAP-1 contribute to high lymphocyte adhesion on the vascular endothelium at the periphery of primitive liver tumors.

This mechanism might increase the immune response to the tumor, while other adhesion molecules involved in the multistep process of adhesion are also high in primary liver tumors. The increased expression of VAP-1 is specific to hepatic tumors because other non-hepatic tumors are not associated with elevated levels of VAP-1. Therefore it may be a molecule that is expressed in hepatic tumor vessels but is down-regulated in some tumors during the growth of the malignant process or not up-regulated in other cases.^{66,67} The underlying liver disease too affects the expression of VAP-1, in particular it has been noticed that patients with HCC associated with a liver disease with alcoholic etiology have higher levels of VAP-1 compared to other patients with HCC. It is generally higher among patients with HCC, while it is not high either among patients with HCC associated HCV or in those who have HCC associated with steatohepatitis, while patients with a diagnosis of cirrhosis from steatohepatitis without HCC have high concentrations. The mechanism is still unclear.⁶⁸

Intercellular Adhesion Molecule 1 (ICAM-1)

ICAM-1 is a highly glycosylated single-chain glycoprotein with a polypeptide core of 55 kD belonging to the immunoglobulin superfamily.^{69,70} The main function of ICAM-1 is to participate in the recognition and adhesion between cells. Its molecular counterpart in this bond is recognized in two proteins: lymphocyte function-associated antigen (LFA-1) and the macrophage surface antigen (Mac-1). ICAM-1 is an essential molecule for the interaction between T lymphocytes and fibroblasts, as well as for the agglutination between lymphocytes and lymphocytes or between lymphocytes and endothelial cells. The expression of ICAM-1 is relatively increased in metastatic tumors. Furthermore along with the cancer cells that express different levels of ICAM-1, lymphocytes with ICAM-1 can be found in almost all cancers. Various cytokines can facilitate its expression through the activation of NF- κ B signaling pathway.

In the healthy liver hepatocytes do not express ICAM-1 while endothelial cells of liver sinusoids and endothelial vascular cells have a weak expression of ICAM-1. In various types of hepatitis the expression of ICAM-1 is increased in endothelial cells of liver sinusoids and in endothelial vascular cells and it is positive also in hepatocytes, cholangiocytes, lymphocytes and fibroblasts. Serum ICAM-1 levels are significantly higher in patients with liver cirrhosis: here it plays a major role, especially in cases where cirrhosis is associated with HCC. In fact most of the cell types present in primitive hepatic tumors are cancer-associated fibroblasts (CAF). They play an important role in neoplastic invasion, angiogenesis and metastasization process. The normal fibroblasts can promote cells' differentiation and inhibit the growth of cancer cells but CAFs, in contrast, can stimulate the tumoral growth and infiltration. The tumor microenvironment full of IL-6 and ICAM-1 expressed by CAFs facilitates metastases. The CAFs activated by pro-inflammatory cytokines such as TNF are an important source of ICAM-1.⁶⁹

The levels of ICAM-1 in patients with HCC are reported to be significantly higher than normal. The expression of ICAM-1 is different between the areas of normal liver, of atypical hyperplasia and of HCC.⁷¹ The expression of ICAM-1 in endothelial and mesenchymal cells is inversely proportional to the degree of atypical hyperplasia, but on the contrary its expression in hepatocytes is significantly higher than in non-neoplastic liver cells. In fact, ICAM-1, besides being secreted by immune cells stimulated by tumor antigens, is also released by the cancer cells themselves, which could be one of the molecular mechanisms of tumor immune escape. The level of ICAM-1, moreover, increases with the stage of the tumor, while it declines after surgical resection.⁶⁹

It has also been discovered how ICAM-1 positive cancer cells are able to induce tumors in vivo, showing that HCC cells expressing ICAM-1 possess properties typical of cancer stem cells. Then the conclusion was reached that ICAM-1 could be used as a potential marker for CSCs. In fact the specific inhibition of ICAM-1 expression in vivo reduces the development of HCC and metastases, suggesting that this molecule could be used as a therapeutic target. The fact that CSCs isolated from the same tumor may express different markers suggested that there are different populations of CSCs, among which the main one would be positive ICAM-1. Moreover, the presence of positive ICAM-1

cancer cells in the blood of patients with HCC provides evidence of the existence of circulating tumor cells (CTC), which are also thought to possess CSC properties, and to be in turn a heterogeneous set of cell populations.⁷²

The expression of ICAM-1 is strongly correlated to the size of the tumour and to the development of metastases. Probably ICAM-1, combining with its natural ligand LFA-1, allows the cancer cells to reach the blood stream along with lymphocytes. Other studies suggest that ICAM-1 may play a role in the disintegration of the cell adhesion local system, in cellular contractions and in the cytoskeletal reconstruction, so as to facilitate the migration of the cells themselves. Moreover, a high expression of ICAM-1 helps the cells separate from each other and move. Finally, it is important for angiogenesis too, in fact along with VCAM-1 it promotes the expression of various cytokines, which in turn encourage the formation of vessel intima. Even isolated tumor endothelial cells (TECs) and normal endothelial cells express ICAM-1 on the surface and are responsible for the formation of capillaries.

The diagnostic and prognostic role of ICAM-1 was also studied. It has been noticed in fact that, in patients with negative AFP, space-occupying lesions have been found 1 to 4 months after the discovery of abnormally high levels of soluble ICAM-1 (> 1000 microg/l). ICAM-1 levels are higher in patients with HCC versus those with benign tumors and normal controls, and its expression increases with the stage of cirrhosis. It is also important in the evaluation of the conditions of HCC patients and of the effects of treatments.^{69,70,73}

Vascular Cell Adhesion molecule 1 (VCAM-1)

VCAM-1 is a transmembrane glycoprotein that belongs to the immunoglobulin superfamily. It is one of the adhesion molecules involved as mediators in angiogenesis. VCAM-1 is expressed transiently in vascular endothelial cells activated in response to VEGF and other cytokines such as TNF, IL-1 β and IFN-gamma. Functionally, its expression plays a crucial role in the adhesion of leukocytes to the endothelium in tissues involved in inflammatory processes and in neoplastic diseases. It also plays an important role in providing an attachment to the endothelium during angiogenesis, as well as acting as an adhesion molecule to facilitate the development of metastases, for

example in melanoma. It is present in soluble form too and as such it can be found in circulation.

VCAM-1 is overexpressed in various neoplastic and non-neoplastic diseases. Some studies have shown high serum levels of VCAM-1 in patients with colorectal cancer, stomach and breast cancer, as well as being an indicator of adverse prognosis in patients with Hodgkin lymphoma.

Increased expression of VCAM-1 has been reported in chronic liver diseases, suggesting that VCAM-1 may play a certain role in the pathogenesis of chronic liver diseases and cirrhosis of the liver. Higher serum levels of VCAM-1 were found in several studies on chronic liver diseases regardless the etiology. The average level of VCAM-1 in patients with HCC is similar to that of healthy controls, however in patients with HCC and cirrhosis the level is higher. Furthermore high serum levels of VCAM-1 have a positive correlation with serum bilirubin levels, and inversely with albumin values and platelet count. This indicates how the levels of VCAM-1 in serum are linked to the severity of the underlying liver disease while, on the contrary, there is no statistically significant correlation between expression of VCAM-1 and tumor size, its pathologic characteristics, stadium or invasiveness. This is in contrast to many other types of cancer where high levels of VCAM-1 are associated with a more advanced stage of malignant disease. Probably in the liver VCAM-1 plays a less important role as a mediator in angiogenesis or other pathological processes in HCC. In fact Yoong et al. have shown how in HCC there are higher levels of VAP-1 and ICAM-1 rather than VCAM-1. The serum level of VCAM-1 in patients with HCC on non-cirrhotic liver is lower compared to normal controls, making it possible to assume that VCAM-1 is down-regulated in patients with HCC. On the contrary, instead, VCAM-1 appears to be important in liver inflammation and in the development of fibrosis, probably by mediating interactions between lymphocytes and endothelium. Finally it has been noticed how lower values of VCAM-1 are associated with a longer disease-free time compared to those with higher values. This is owing, more than to HCC characteristics, to the fact that higher levels are due to a worse liver disease, which in turn correlates positively with a greater risk of relapse.⁷⁴⁻⁸¹ In contrast to this study, other researchers have proposed instead a role of VCAM-1 in the development of HCC. For example, Hong Li et al. have noticed how

VCAM-1 is an important mediator in the signaling pathway going from the activation of HIF-1 α to the expression of angiopoietin-like protein 4 (ANGPTL4), which promotes, among its varied and complex tasks, angiogenesis in HCC. Finally, Song et al. and Alexander et al. too have shown the role of VCAM-1, together with ICAM-1, in angiogenesis in ongoing HCC.⁸²

AIMS

The main aim of the study is to evaluate IL-6, TNF, VAP-1, ICAM-1 and VCAM-1 expression in serum samples of cirrhotic patients with and without HCC, thereby giving an interpretation of the role played by inflammation in the carcinogenetic process. Secondary endpoints are the identification of prognostic markers and future therapeutic targets.

MATERIALS AND METHODS

Eighty consecutive patients with cirrhosis followed as outpatient controls between 2002 and 2012 at Azienda Ospedaliera di Padova were included. Twenty-two patients were affected by alcoholic cirrhosis, while fifty-eight presented liver cirrhosis and HCC. In the group of patients with HCC, forty-four patients underwent loco-regional therapies, whereas fourteen underwent surgery.

Evaluation of cirrhosis was carried out by Child-Pugh, MELD and MELD-Na staging systems (tab. 2 to 4), ascites, encephalopathy, α -fetoprotein levels (α FP), tumour size and overall survival in HCC patients. A group of 20 healthy patients was used as control group. All patients were checked for the presence of diabetes.

The evaluation of soluble VAP-1 serum levels (sVAP-1) was performed by Enzyme Linked Immunosorbent Assay (*ELISA - Bender MedSystems GmbH*) carried out on plasma samples after defrosting. The evaluation of the inflammatory cytokines (IL6 and TNF- α) and of the adhesion soluble forms of cell adhesion proteins (ICAM-1 and VCAM-1) was performed by Bio-Plex system (*kit Bio-Plex Pro™ kit - Bio-Rad*) carried out on plasma samples after defrosting.

Numerical data were expressed as mean and standard deviation and compared with Student's t-test, while for categorical variables count and percentage in each category are reported and compared with Wilcoxon test. Cox regression and KaplanMeier method were performed for the survival study. Statistical significance was declared for P-value <0.05.

The statistical analysis was performed with STATA/SE 11.1 (*StataCorpLP, College Station, Texas, USA*).

RESULTS

This study included 80 patients followed through outpatient controls between 2002 and 2012. The mean follow-up was 5 years. Patients were divided into 3 groups: 22 cirrhotic patients without HCC, 44 cirrhotic patients with HCC who underwent loco-regional therapies, and 14 cirrhotic patients with HCC who underwent surgical procedures.

Tab. 1 staging of cirrhosis in the three groups

Group	Patients	Therapy	Sex	Age	Child A	Child B	Child C
Cirrhotic patients	22	Medical Therapy	13 male 9 female	54 (43-77)	3	11	8
					MELD ≤13 7	MELD 13-19 9	MELD ≥19 6
					MELD-Na ≤13 5	MELD-Na 13-19 9	MELD-Na ≥19 8
Cirrhotic patients + HCC	44	Loco-regional therapies	34 male 10 female	62 (35-89)	22	15	7
					MELD ≤13 29	MELD 13-19 11	MELD ≥19 4
					MELD-Na ≤13 25	MELD-Na 13-19 10	MELD-Na ≥19 13
Cirrhotic patients + HCC	14	Surgical procedures	13 male 1 female	63 (38-83)	12	2	0
					MELD ≤13 14	MELD 13-19 0	MELD ≥19 0
					MELD-Na ≤13 14	MELD-Na 13-19 0	MELD-Na ≥19 0

In the first group 13 were male and 9 females. The mean age was 54 years (range 43-77). 3 patients were Child A (13,6%), 11 Child B (50%) and 8 Child C (36,4%). 7

patients were MELD ≤ 13 (31,8%), 9 between 13 and 19 (41%), 6 patients >19 (27,2%). 5 patients has a MELD-Na ≤ 13 (22,7%), 9 between 13 and 19 (41%), 8 patients >19 (36,3%).

In the second group 34 were males (77,3%) and 10 females (22,7%). The mean age was 62 years (range 35-89). 22 patients were Child A (50%), 15 Child B (34,1%) and 7 Child C (15,9%). 29 were MELD ≤ 13 (66%), 11 between 13 and 19 (25%), 4 patients >19 (9%). 25 patients has a MELD-Na ≤ 13 (56,8%), 10 between 13 and 19 (22,7%), 13 patients >19 (20,5%).

In the third group 13 were males and 1 female. The mean age was 63 years (range 38-83). 12 patients were Child A (85,7%) and 2 Child B (14,3%). All patients have a MELD and MELD-Na ≤ 13 [Tab. 1].

Glycemia was checked to exclude diabetes when blood sugar level was less than 5,6 mmol/l.

All patients were studied with abdominal ultrasound, abdominal CT scan wit contrast or MRI with hepatospecific contrast, thorax CT scan with contrast, bone scintigraphy. All patients who underwent surgery were also studied with an intraoperative liver ultrasound. Surgery was radical (R0) in all patients.

First, a comparison between cirrhotic patients without HCC and healthy controls was carried out. A statistical significant difference was found comparing the mean value of each pro-inflammatory molecule between the two groups ($p < 0.05$).

Once proved the difference between cirrhotic patients and healthy controls, the evaluation of the parameters was carried out comparing cirrhotic patients without HCC to those with HCC.

VAP-1 ($p=0.0005$) and VCAM-1 ($p=0.001$) were significantly higher in cirrhotic patients without HCC compared to cirrhotic patients with HCC [fig. 5-6]. No statistical difference was assesed in levels of the other parameters α FP, TNF- α , IL6 and ICAM.

[Tab. 2]

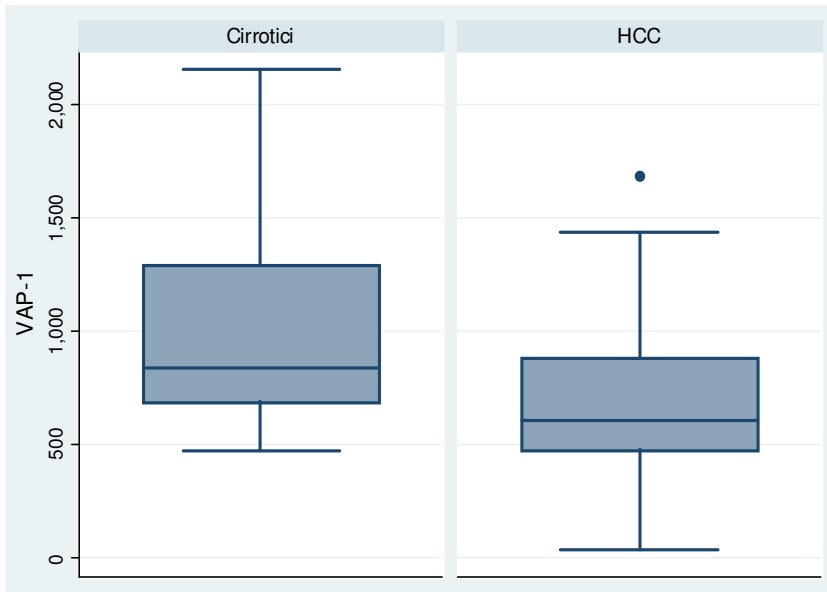


Fig. 5 Difference in VAP-1 expression between cirrhotics patients with and without HCC

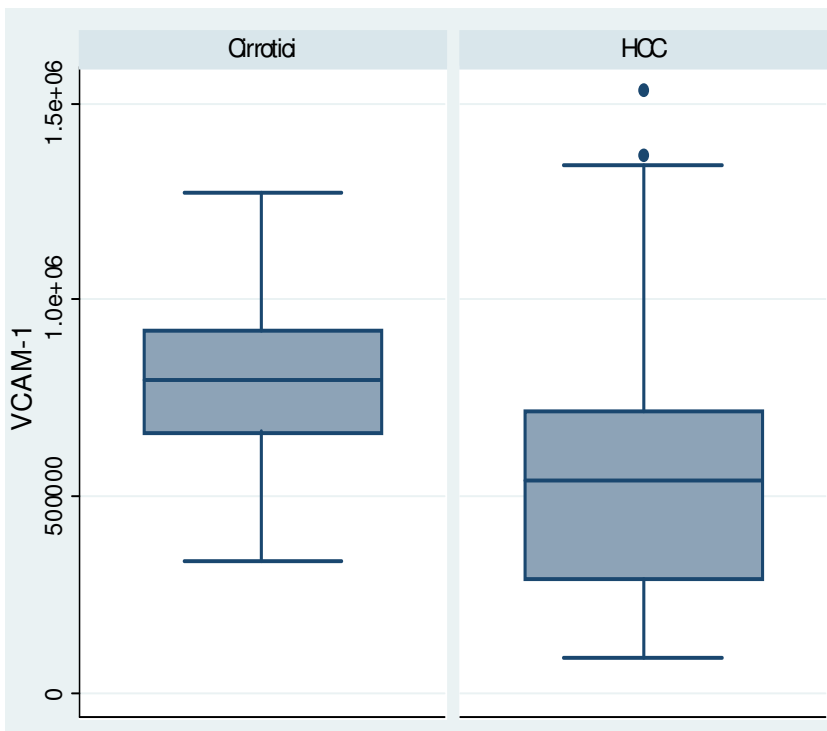


Fig. 6 Difference in VCAM-1 expression between cirrhotics patients with and without HCC

Tab. 2: comparison of values in cirrhotic patients with and without HCC

Parameter	Mean value in cirrhotic patients	Mean value in cirrhotic patients with HCC	P value
IL6	13.85	12.12	0.702
TNF α	5.37	6.93	0.348
VAP-1	1033,30	683,60	0.001
ICAM-1	369942	373875	0.960
VCAM-1	797566	572553	0.005
α FP	2,61	3601,00	0.455

The survival analysis has demonstrated a mean 5-years survival of 47% and a median life expectancy of 40 months (fig. 7).

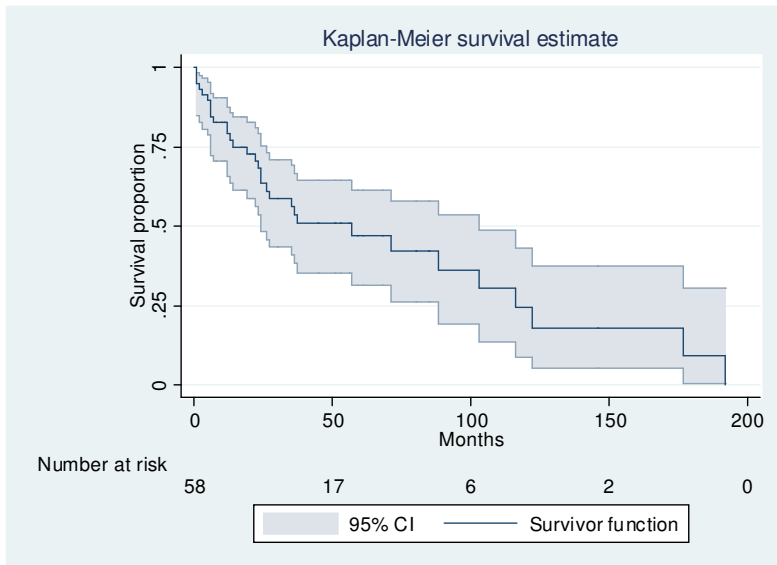


Fig. 7 Kaplan-Meier curve of patients with HCC who underwent surgery or locoregional therapy

The group of patients treated with medical therapy showed a mean of 5-years survival of 42% and a median life expectancy of 38 months (fig. 8), while surgical patients showed a mean 5-years survival of 55% and a median life expectancy of 97 months (fig. 9).

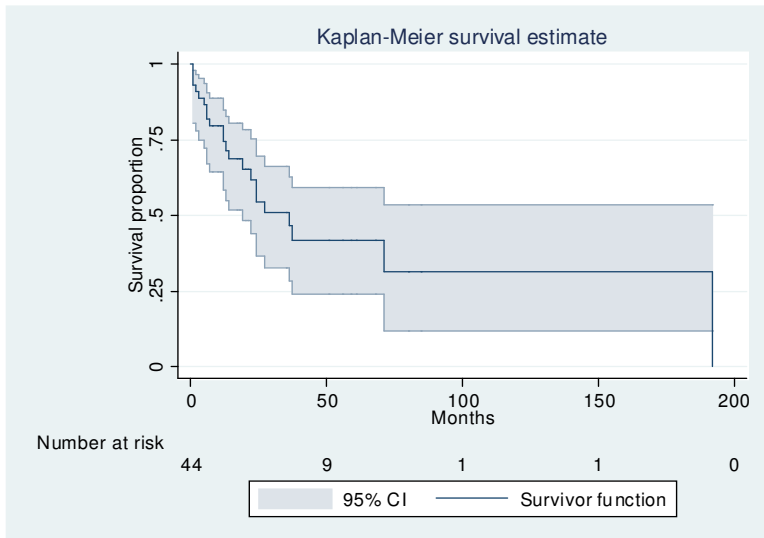


Fig. 8 Kaplan-Meier curve of patients with HCC who underwent locoregional therapy

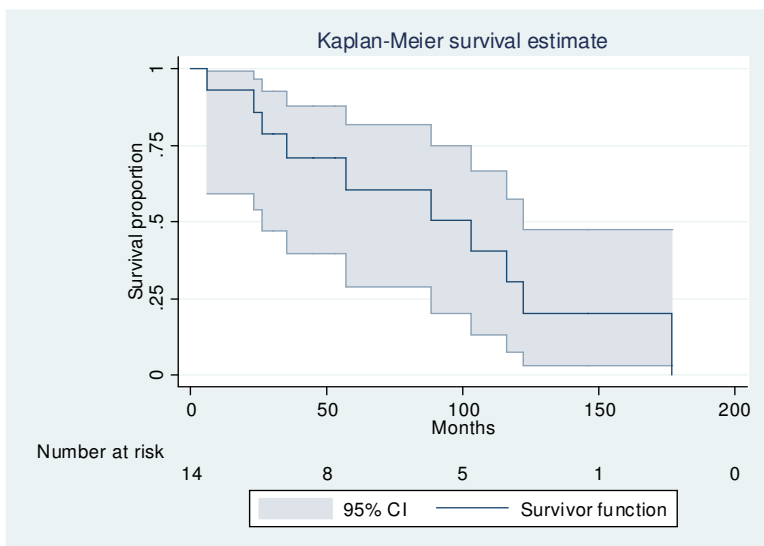


Fig. 9 Kaplan-Meier curve of patients with HCC who underwent surgery

The univariate analysis has demonstrated a negative correlation between prognosis and the following parameters: ascites and tumor size. The death risk among those patients with ascites was 2.7 times higher compared with those without, suggesting ascites as a marker of poor prognosis ($p=0.012$). Also tumour size was shown as a marker of poor prognosis ($p=0.027$), with an HR of 1.01. No statistical significance was found at univariate analysis for the other parameters (IL-6, TNF- α , VAP-1, ICAM-1, VCAM-1, Child-Pugh, MELD, MELD-Na, Encephalopathy, α FP). [tab. 3]

Tab. 3 Overall Survival at univariate analysis

Parameter	Hazard Ratio	CI 95%	P value
IL6	0.98	0.958 - 1.01	0.404
TNF- α	0.95	0.873 - 1.03	0.242
VAP-1	0.999	0.998 - 1	0.317
ICAM-1	1.000001	0.999 - 1	0.342
VCAM-1	1	0.999 - 1	0.83
Child-Pough	2.51	0.445 - 5.716	0.086
MELD	1.03	0.966 - 1.104	0.341
MELD-Na	1.04	0.983 - 1.103	0.166
Encephalopathy	2.24	0.840 - 6.003	0.107
Ascites	2.702	1.246 - 5.858	0.012
Tumor Size	1.01	1.001 - 1.019	0.027
α FP	1	0.9999 - 1	0.306

At multivariate analysis, tumor size and ascites were shown to be statistically significant, with a p respectively of 0.033 (HR 1.01 CI 95% 1.00083-1.02) and 0.02 (HR 2.54 CI 95% 1.159-5.57).

DISCUSSION

The inflammatory process has a complex role in cancer onset since it could both help cancer development, and activate immune response. Several studies have already evaluated the role of cytokines in cancer onset, even in HCC. These molecules could be divided in Th1 cytokines, that promote the induction of a pro-inflammatory response, and Th2 cytokines, which are involved in immune suppression as anti-inflammatory molecules. As Th2 anti-inflammatory cytokine IL6 should lead to the shift of Th1/Th2 balance towards a Th2 response, thus suppressing immunity against cancer and promoting cell proliferation. Actually in the literature some authors have reported that IL6 promotes cell proliferation via STAT3 activation, while others described IL6 with both Th1 and Th2 functions, resulting in a promotion of the inflammatory process and of immune surveillance on tumor. As the latter hypothesis describes, in this study IL6 although without a statistical significance has higher values in cirrhotic patients without HCC than in those with cancer, likely suggesting a role in immune surveillance.

TNF- α exerts its role as pro-inflammatory cytokine by activating NF- κ B and the resulting cascade of events, with a cytoprotective effect. Instead in case of advanced cirrhosis the same events allow neoplastic cells survival through the activation of the TNF- α /ROS/HIF-1 α induced by FoxM1 overexpression. Thus, TNF- α can both promote and counteract cancer onset.

Although TNF- α was reported at higher concentrations in HCC patients, in this study the data were not consistent to those reported in the literature

Adhesion proteins allow the passage of leukocytes from blood to hepatic parenchyma. They are obviously more represented in a inflammatory context, while their reduction leads to a lower number of tumour infiltrating lymphocytes (TIL), which are fundamental for immune surveillance. Indeed, in the literature VAP-1 is reported at higher concentrations in inflammatory pathology, while its levels are lower in several cancers.

HCC mainly grows in an inflammatory microenvironment as cirrhosis, thus leading to various hypotheses concerning the vascular adhesion molecules. Some authors have reported VAP-1 at higher levels in HCC patients compared to those with cirrhosis only, while in this study VAP-1 has lower concentrations in HCC patients as the literature

describes in case of other cancer types. Thus, it can be assumed that the antitumor effect of VAP-1 is more important than its pro-inflammatory function, hypothesizing that VAP-1 is primarily involved in immune escape through TIL reduction.

Similarly to VAP-1, the pathogenic mechanism of ICAM-1 seems to be the loss of cancer immunosurveillance but with an opposite trend of the concentration of these two molecules in HCC patients. Even if in the literature the level of ICAM-1 is reported to be higher in HCC patients and to be negatively correlated with prognosis, in this study these data weren't confirmed.

The hypothesis to be confirmed in future studies is that, since ICAM-1 is expressed in both immune and cancer cells, it is likely that its upregulation in the neoplastic clone allows this to hide itself in healthy tissues, thus leading to the immune escape.

The literature describes that VCAM-1 has higher levels in several tumors, but its carcinogenic role is still unclear. Some authors hypothesize that VCAM-1 is pivotal in liver diseases and cirrhosis rather than in HCC. Indeed, its level correlates mainly to the stage and aetiology of cirrhosis, as confirmed by this paper, supporting the hypothesis that its main role is in the inflammatory process and not in carcinogenesis.

The analysis of these cytokines and adhesion molecules point out how complicated is the interaction between inflammatory and cancerogenic processes. These molecules have conflicting roles. They act in response to the microenvironment caused by chronic liver damage and cirrhosis, thus stimulating cell cycle progression in response to the continuous damage-death-regeneration of cells with a resulting dysregulation of cell cycle and subsequent selection of neoplastic clones. Moreover, cancer clones are able to take advantage of the same molecules, hiding themselves into healthy tissues as suggested mainly by the overexpression of ICAM described in the literature. On the other hand, they are pivotal for activation and migration of the tumor infiltrating lymphocytes and the subsequent host immune response against cancer.

Looking to the prognostic value of the cytokines and vascular adhesion protein, this study didn't find any statistical significance between groups, and also in the literature data on the prognostic values of these molecules are still conflicting. Actually, as described in this study, only the clinical parameters have a prognostic value.

LIMITS AND FUTURE AIMS

The preliminary results are not conclusive and further studies are needed to clarify the intriguing role of cytokines and vascular adhesion protein in carcinogenesis and their prognostic value.

The main limit of this paper is the lack of uniformity of the population studied. The different rate of staging subgroups in the various population may have affected the statistical analysis.

A prospective case-control study is the next postdoc research topic. The first endpoint will be the homogenization of the population to be studied, thus allowing the evaluation of patients by stage (Child-Pough; Meld) and by etiology of cirrhosis (alcoholic, HBV, HCV), and furthermore making a comparison between the different categories and subgroups. Furthermore, as secondary endpoint, the study will focus on finding potential therapeutic targets and the subsequent therapeutic implication.

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