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Environmental risk factors in primary liver cancer: a review of the literature and perspectives for primary prevention and early detection

Fattori di rischio ambientali nei tumori epatici primitivi: revisione della letteratura e prospettive per la prevenzione primaria e la diagnosi precoce

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Summary

Primary liver cancer is a major public health problem, accounting for about 600,000 deaths in the world annually, with hepatocellular carcinoma (HCC) accounting for about 80% of all primary tumours in the liver and intra-hepatic cholangiocarcinoma (ICC) representing about 10-15% of the remaining primary hepatic malignancies. Incidence and mortality trends for both HCC and ICC are increasing globally, with particular concern for US and Europe, and survival rates are still very poor. Some risk factors are well-established, such as hepatitis viruses, alcohol intake and aflatoxins exposure for HCC, and infection with liver flukes and primary sclerosing cholangitis for cholangiocarcinoma (CC). However, these known etiologies do not explain the observed increased incidence of

Riassunto

I tumori primitivi maligni del fegato sono un grande problema di salute pubblica, essendo la causa di 600.000 decessi l'anno nel mondo. Il carcinoma epatocellulare (HCC) rappresenta l'80% di tutti i tumori epatici maligni primitivi, mentre il colangiocarcinoma intra-epatico (ICC) rappresenta circa il 10-15% dei rimanenti tumori epatici maligni. L'incidenza e l'andamento della mortalità per HCC e ICC sono globalmente in aumento, con particolare preoccupazione negli Stati Uniti e in Europa, e i tassi di sopravvivenza sono ancora molto bassi. Alcuni fattori di rischio sono ben stabiliti: i virus dell'epatite, il consumo di alcool e l'esposizione ad aflatossine per l'HCC; le parassitosi da vermi epatici e la colangite sclerosante primaria per il colangiocarcinoma (CC). Tuttavia, queste eziologie conosciute non spiegano l'osservato

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these two malignancies worldwide. Environmental carcinogens could play an underestimated rôle in the increased global burden of primary liver cancers. An essential tool to identify chemical and physical carcinogenic agents is long-term carcinogenicity bioassays, such as those performed by the US National Toxicology Program and the Ramazzini Institute. Although some strains of rodents are reported to be more susceptible to chemical-induced hepatocarcinogenicity, many similarities in histologic, cytological and molecular pathways are shared between humans and rodents in liver tumorigenesis, thus making long-term carcinogenicity bioassays the best available tool for identifying environmental carcinogenic agents, including those targeting the liver. Moreover, early detection of HCC and CC should provide a valuable means to decrease mortality rates in the short term, but reliable biomarkers are not yet available for clinical practice. Advanced technologies such as proteomics and *in vivo* imaging techniques are now available for animal cancer models. Well-designed protocols which will integrate a proteomic approach with imaging diagnostics using animal models may result in greater improvement for biomarkers development in early diagnosis of primary liver cancers. Eur. J. Oncol., 14 (3), 133-150, 2009

Key words: **hepatocellular carcinoma, cholangiocarcinoma, environmental carcinogens, long-term carcinogenesis bioassays, rodents, biomarkers**

aumento di incidenza di questi due tumori maligni nel mondo. I cancerogeni ambientali potrebbero giocare un ruolo sottostimato nell'aumentata incidenza globale dei tumori primitivi del fegato. Uno strumento essenziale per identificare agenti cancerogeni chimici e fisici sono i saggi di cancerogenicità a lungo termine, come quelli effettuati al National Toxicology Program degli Stati Uniti e all'Istituto Ramazzini. Nonostante sia riportato in letteratura che alcuni ceppi di roditori siano più suscettibili all'insorgenza di epatocarcinomi chimicamente indotti, molte somiglianze istologiche, citologiche e nei meccanismi molecolari sono condivise negli esseri umani e nei roditori per quanto riguarda la cancerogenesi epatica, rendendo così i saggi di cancerogenicità a lungo termine il miglior strumento disponibile per identificare gli agenti cancerogeni ambientali, inclusi quelli che colpiscono il fegato. Inoltre, l'identificazione precoce di HCC e CC potrebbe fornire un mezzo importante per diminuire i tassi di mortalità nel breve periodo, ma nella pratica clinica non sono ancora disponibili biomarcatori affidabili. Tecnologie avanzate, come la proteomica e la diagnostica per immagini *in vivo*, sono ora disponibili per i modelli animali del cancro. Protocolli ben progettati, che integrino un approccio proteomico con la diagnostica per immagini utilizzando modelli animali per il cancro, possono contribuire a un importante miglioramento per lo sviluppo di biomarcatori per la diagnosi precoce dei tumori primitivi maligni del fegato. Eur. J. Oncol., 14 (3), 133-150, 2009

Parole chiave: **carcinoma epatocellulare, colangiocarcinoma, cancerogeni ambientali, saggi di cancerogenicità a lungo termine, roditori, biomarcatori**

Abbreviations

AFB1: Aflatoxin B1
AFP: Alpha-fetoprotein
AHF: Altered hepatic foci
AUROC: Area under receiver operating curve
CC: Cholangiocarcinoma
CT: Computed Tomography
CYP4502E1: Cytochrome P450 2E1
ECC: Extrahepatic cholangiocarcinoma
ER: Estrogen Receptor
HCC: Hepatocellular carcinoma
HBV: Hepatitis B Virus
HCV: Hepatitis C Virus
HTLV-1: Human T-cell lymphotropic virus
ICC: Intrahepatic cholangiocarcinoma
MRI: Magnetic Resonance Imaging
NAFLD: Non-alcoholic fatty liver disease
PAH: Polycyclic aromatic hydrocarbons
PET: Positron Emission Tomography
PSC: Primary sclerosing cholangitis
VCM: Vinyl chloride monomer

Introduction

Primary liver cancer is a major public health problem, accounting for about 600,000 deaths in the world in 2002 (1). Hepatocellular carcinoma (HCC) accounts for about 80% of all primary cancers in the liver, while intra-hepatic cholangiocarcinoma (ICC), arising from the epithelium of bile ducts located in the liver, represents about 10-15% of the remaining primary hepatic malignancies (2, 3). Hepatoblastoma (a malignant embryonal tumour of the childhood) and hepatic angiosarcoma (arising from blood vessels) account for the remaining 5-10% (2).

In this review we will summarize current epidemiological data on HCC and biliary malignancies, particularly ICC. Biological, dietary and environmental risk factors of these diseases will be reviewed. The rôle of experimental carcinogenicity bioassays in the identification of hepatocarcinogens and of animal models for development of biomarkers for early detection of carcinogenic process in the liver will be discussed.

Epidemiology

Geographic distribution

HCC is the fifth most common cancer worldwide with about 500,000 new cases annually, representing the third most common cause of cancer-related death among men and the eighth in women (1, 4). The geographic distribution of primary liver cancer burden is not even: more than 80% of HCC cases occur in sub-Saharan Africa or Eastern Asia (5) with China accounting for 55% of the world's cases (2). Cholangiocarcinoma (CC) has highest incidence rates in some Asiatic countries, such as Thailand with 96 cases per 100,000 people, about 100 fold with respect to some Western countries (6).

Incidence and mortality trends

Time trends in primary liver cancers may be difficult to interpret due to changes in classification, variable inclusion of metastatic tumours and only recent inclusion of topographical coding for CC distinguishing intrahepatic from extrahepatic CC. However, slight decreases in the HCC incidence in high-rate areas, such as China and Japan, have been reported (7), while a sustained increasing trend has been reported in low-rate countries, particularly the United States and some European countries, such as Italy, France, UK and Germany (2, 5). It merits noting that these increasing trends are associated with younger age groups (2). In the United States HCC incidence rates doubled in the period 1985-2002 with age distribution shifting towards younger age ranges, particularly 45-60 years old, and HCC has become the fastest growing cause of cancer-related death in men (8). Interestingly, it has been reported that in the United States 15-50% of HCC patients had no established risk factors, such as hepatitis viral infections, heavy alcohol consumption or aflatoxin B1 exposure (5). In Europe an analysis of mortality rates from HCC trends in the last 20 years has shown increasing rates for men in 11 countries and for women in 6 countries out of 17 whose data were considered (9). In particular, France and Italy are the countries with the highest mortality rates from HCC for men in the period 2000-2003, with 6.79 and 6.72 deaths/100,000 respectively. In the last 20 years mortality rate per 100,000 men has increased from 3.57 to 6.79 in France and from 5.60 to 6.72 in Italy. Italy is also the first country for mortality rates from HCC for women (1.92 deaths/100,000), while France has 0.96 deaths/100,000. However, mortality rates for women show a decrease from 2.48 to 1.92 per 100,000 women in Italy and an increase from 0.69 to 0.96 per 100,000 women in France in the last same period (9).

With regard to biliary malignancies, ICC and extra hepatic CC (ECC) show different epidemiologic features: ICC incidence and mortality are increasing worldwide, while those of ECC are slightly decreasing (3). A US study using data from the Surveillance, Epidemiology and End Results (SEER) program has shown that incidence trends in ICC more than doubled approximately between 1976 and 2000 (10). Similar results have been shown in another study which reported a 165% increase in ICC incidence comparing the period 1975-1979 to 1995-1999 (6). ICC incidence and mortality rates are also increasing in most European countries: in the last decades trends in mortality rates show an

increase in 13 out of 15 analyzed countries for both men and women (11). In particular England-Wales and Scotland showed the highest mortality rates for both men and women: 0.83 (men) and 0.63 (women) deaths/100,000 and 1.17 (men) and 1.00 (women) deaths/100,000 respectively. The percent increase is 315% for men and 271% in women in England-Wales comparing 1979-1981 with 1995-1997, while in Scotland this increase is 216% for men and 335% for women in the same period (12).

Survival

Survival rates for HCC after 5 years are still very poor also in developed countries, as low as 8% in the United States, 10% in Italy and 9% in France (2, 13, 14). Survival rates for CC are also very poor, ranging 2-7%, although early diagnosis may allow surgical resectability of small or little infiltrating malignancies, thus increasing 5-years survival rates to 25-30% (15-18).

Biological risk factors

Hepatitis B (HBV) and C (HCV) infections have been reported to be associated with over 80% of HCC in the world (19). It has been estimated that HBV carriers have more than 100-fold increased risk of HCC compared with non-infected individuals (20). HCV infection is a more important risk factor for HCC than HBV in Western countries and Japan. HCV infection markers have been detected in variable proportions of patients in Italy (44-66%) (21), France (27-58%) and Spain (60-75%). HCV-associated HCCs have been reported as high as 80-90% in Japan (22). Different mechanisms of carcinogenesis are associated to HBV and HCV infections. HBV is a known oncogenic virus, able to integrate its DNA into the genome of the infected cells causing direct mutagenesis, and higher rates of chromosomal abnormalities have been found in HBV-related HCC (23, 24). HCV virus seems to cause HCC through an indirect pathway causing chronic inflammation, cell death, proliferation and cirrhosis (20). However, HBV virus also seems to promote liver carcinogenesis through indirect pathways by continuous hepatocyte injury, liver regeneration and tumour-promoting activities of viral proteins, like Hbx (20). It is interesting to note that a recent epidemiological study (25) found an association between a leukemogenic virus infection, HTLV-1, and an increased risk of HCC (RR=2.1; 95% C.I: 1.0-4.6). Although confounding factors due to interactions between HCV and HTLV-1 infections has been pointed out as a partial explanation of these data, further studies are warranted to explore these findings.

Etiology and risk factors for CC are much less known compared to HCC ones. Some inflammatory diseases of biliary or gastrointestinal tract are associated with an increased risk of CC, notably gallstones, chronic ulcerative colitis and primary sclerosing cholangitis (PSC) (26, 27). PSC has been indicated as the most common cause of CC in Western countries, but since its incidence has not increased in the last decade it cannot explain the observed increased incidence of CC (3, 6). In Eastern Europe and in many Asian countries an important etiologic factor of CC is infection with liver flukes (*Opistorchis spp*., *Clonorchis sinensis*) which contaminated raw food, particularly fish (28). A 1994 WHO estimate reported about 17 million people globally infected with CC-related liver flukes (29). HCV infection was also associated with higher risk of developing ICC in the United States (30), but still most patients with CC have no identifiable risk factors for the disease.

Dietary risk factors

Excessive alcohol intake (more than 3 drinks/day corresponding roughly to 35-40 g ethanol/day) has been recognized as human carcinogen by IARC in 1988 (31). Ethyl alcohol is a multipotent carcinogenic agent and has been causally associated not only to liver cancer, but also to other cancer sites (oral cavity, pharynx, larynx, esophagus, liver, colorectum and female breast) (32). Ethyl alcohol in drinking water has also been demonstrated to be carcinogenic in experimental animals (33).Various mechanisms seems to act in alcohol-associated carcinogenesis: chronic inflammation resulting in oxidative stress, as in steatohepatitis; acetaldehyde formation (by liver alcohol dehydrogenase and

cytochrome P4502E1, CYP2E1) and its consequent mutagenic and carcinogenic effects; induction of CYP2E1 causing increased reactive oxygen species (ROS) formation and subsequent lipid peroxidation and DNA damage; depletion of cellular antioxidant defense pool (glutathione and alpha-tocopherol); disturbed methyl group transfer associated with DNA hypomethylation; decrease retinoic acid in the liver with induction of AP-1 complex, the latter being a downstream effector of tumour promoters, oncogenes and growth factors; and iron overload, due to increased intestinal uptake and hepatic deposition, promoting ROS formation through the Fenton reaction (34).

Aflatoxins are known and potent hepatocarcinogens in both animals and humans (35). These mycotoxins are produced by *Aspergillus flavus* and related fungi on improperly stored corn, rice and peanuts. Sub-Saharian and Eastern/Southeastern populations are highly exposed (2). Little information is available on correlation between aflatoxins and HCC in developed countries, but it merits noting that aflatoxins adducts have been identified in a small sample of patients in the United States (36). Many ecological studies performed between the 1960s and 1980s showed aflatoxins induced HCC in exposed populations (37-39). Many studies in rats have also demonstrated the carcinogenicity of aflatoxins: a dose-related increased of hepatomas were firstly shown by Newberne (40). Hepatocarcinogenicity of aflatoxins was later confirmed by several studies in different rodent species and strains (39, 41, 42). Aflatoxin B1 (AFB_1) is the standard reference for mechanistic studies on aflatoxins carcinogenicity. AFB_1 is metabolically activated in the liver by cytochrome P450 oxidation, forming the AFB_1 exo-8,9-epoxide that reacts covalently with DNA and also with proteins (43). The most frequently observed mutation induced by metabolically activated AFB_1 is GC→TA transversion. In particular, in the specific case of AFB_1 -induced HCC, a striking sequence specificity has been observed as most of the mutations are found in the third position of codon 249 of p53 gene (44). AFB_1 reactivity is particularly strong towards guanine bases although it also depends on local DNA sequence (45-46). It is also speculated that the observation of the hotspot mutation site in the p53 gene is due to a selective advantage for growth of already mutated hepatocytes, as it has been suggested that AFB_1 exert a major effect on late-stage carcinogenesis (46).

Recently, non-alcoholic fatty liver disease (NAFLD), a metabolic disorder characterized by steatosis, non-alcoholic steatohepatitis (NASH) frequently leading to fibrosis and finally evolving in cirrhosis, has been proposed to increase HCC risk (47). However, in an experimental model of NAFLD, mice exposed to particulate matter PM<2.5 μm develop more severe liver inflammation and fibrosis compared to non exposed mice (48). It is worth noting that in a series of 105 consecutive patients in the United States, cryptogenic cirrhosis accounted for 29% of diagnosed HCC and NAFLD might underlie 13% of total HCC patients for whom neither HCV/HBV infections nor heavy alcohol intake is reported (49). NAFLD is strongly associated with caloric overconsumption, obesity and diabetes, the latter of which is found to be a risk factor for both chronic liver disease and HCC (4, 50).

Concerning CC, few data on dietary risk factors have been reported. Excess alcohol consumption was recently found higher in ICC compared to controls in a case-control study (51). Further experimental and epidemiologic research on food contaminants such as additives or pesticides is warranted to analyse diet influences on CC onset.

Chemical risk factors

An analysis of the data reported by the IARC monographs on the 108 agents classified as human carcinogens (class 1) shows that 17 (about 16%) are causally associated or suspected hepatocarcinogens (Table 1). Among these, viruses such as HBV, HBC and HTLV-1 have already been discussed briefly, as the examples of ethanol in alcoholic beverages and aflatoxins exposure in contaminated foods.

Different classes of chemicals are reported to induce HCC in humans: drugs or hormonal therapies (azathioprine, tamoxifen and estrogen-progesteron oral contraceptives) (68); radioisotopes or heavy metals (Plutonium-239, Radium-224, Thorium-232; arsenic in drinking water) (57, 65); complex mixtures of PAH and other combustion products

Table 1 - Chemical, physical and biological agents recognized or suspected as hepatocarcinogens to humans

Agent/mixture/ exposure situation	Type of agent	Liver cancer (level of evidence)	Other cancer sites in humans	Data on rodent carcinogenicity		References
				Liver	Other sites	
Azathioprine	Immunosuppressant drug	Causally associated	Hematopoietic tissue, skin	Not reported	Hematopoietic tissue, ear duct	IARC Suppl. 7 (52), Cohen *et al* (53)
Estrogen-progesteron oral contraceptives	Hormones	Causally associated	Female breast, cervix	Causally associated	Mammary gland, uterus	IARC Vol 91 (54)
Ethanol in alcoholic beverages	Alcohol	Causally associated	Oral cavity, pharynx, larynx, esophagus, colorectum, female breast	Causally associated	Head (osteosarcomas), neck, mammary gland	IARC Vol 96 (32); Soffritti *et al* (33)
Hepatitis B virus	Virus	Causally associated	Not reported	Suspected to be associated[a]	Not reported	IARC Vol 59 (55)
Hepatitis C virus	Virus	Causally associated	Not reported	Not reported	Not reported	IARC Vol 59 (55)
Human T-cell lymphotropic virus type I	Virus	Suspected to be associated	Adult T-cell leukemia/ lymphoma, cervix, vagina	Not reported	Not reported	IARC Vol 67 (56)
Plutonium-239	Alpha particle emitter	Causally associated	Lung, bone sarcoma	Causally associated	Lung, bones	IARC Vol 78 (57)
Radium-224	Alpha particle emitter	Causally associated	Bone sarcoma, female breast, kidney	Not reported	Bones, hematopoietic tissue	IARC Vol 78 (57)
Thorium-232	Alpha particle emitter	Causally associated	Leukemia	Causally associated	Bones	IARC Vol 78 (57)
Tamoxifen	Hormone agonist/ antagonist	Suspected to be associated	Endometrium	Causally associated	Uterus	IARC Vol 66 (58); Maltoni *et al* (59)
2,3,7,8-tetrachlorodi-benzo-para-dioxin	Organochlorine	Suspected to be associated	Lung, hematopoietic tissue, soft tissues sarcomas, all cancers combined	Causally associated	Thyroid, subcunateous and hematopoietic tissue	IARC Vol 69 (60), Walker *et al* (61)

(continued)

Table 1 - *(continued)*

Agent/mixture/ exposure situation	Type of agent	Liver cancer (level of evidence)	Other cancer sites in humans	Data on rodent carcinogenicity		References
				Liver	Other sites	
Vinyl chloride	organochlorine	Causally associated	Suspected brain, hematopoietic tissue, melanoma	Causally associated	Skin, mammary gland, Zymbal gland, lung, kidney	IARC Vol. 97 (62)
Aflatoxins	Mycotoxins	Causally associated	Not reported	Causally associated	Not reported	IARC Vol. 82 (63)
Betel quid with or without tobacco	Plant derivatives	Suspected to be associated	Oral cavity, pharynx, esophagus	Suspected to be associated	Skin, lung, stomach	IARC Vol. 85 (64)
Soots	PAH and combustion products mixtures	Causally associated	Esophagus, lung, leukemia	Not reported	Skin, lung	IARC Suppl 7 (52)
Arsenic in drinking water	Heavy metal	Suspected to be associated	Skin, lung, kidney, urinary bladder	Causally associated	Skin, lung, urinary bladder	IARC Vol. 84 (65), Waalkes *et al* (66)
Tobacco smoking and tobacco smoke	PAH and combustion products mixtures	Causally associated	Oral and nasal cavities, esophagus, pharynx, larynx, lung, stomach, pancreas, kidney, urinary tract, cervix, hematopoietic tissue	Suspected to be associated	Skin, respiratory tract, lung	IARC Vol. 83 (67)

[a] results were obtained in transgenic mice expressing pre-S, S and X genes of HBV genome and the relevance of these studies is not clear

(soots and tobacco smoking) (52, 67); organochlorines (vinyl chloride monomer: VCM) (62); and plants (betel or *Areca catechu*) (64). For many of these compounds carcinogenicity studies on rodents anticipated or confirmed specific liver tumour induction. It is interesting to note that recently also some psychoactive substances, like cannabinoids, have been reported to worsen liver steatosis and fibrosis in presence of HCV infections (69-71). Despite that no evidence of carcinogenicity has been shown for delta 9-tetrahydrocannabinol (the principal psychoactive ingredient in marihuana) in rats and mice (72), more research is warranted to assess long-term carcinogenicity effects of cannabinoids, particularly in the liver, as their assumption during cannabis smoking may result in cannabinoids exposure for a large population. Moreover, some natural and synthetic cannabinoids have been recently tested for therapeutic applications, such as control of chemotherapy-induced nausea and vomiting in cancer patients (73, 74), appetite stimulation for AIDS patients (74), and control of mood disorders (75). Thus, evaluation of potential cannabinoids carcinogenicity should be addressed to verify their safety as drugs.

Estrogen-progesteron oral contraceptives have been shown to induce liver tumours in women in many case-control studies (54). Carcinogenicity studies on rats showed that oral administration of ethinyl estradiol plus norethisterone to male and female rats induced increased incidence of liver adenomas in treated males and HCC in treated females (54) and that oral administration of mestranol plus norethisterone induced increased

incidence of liver adenomas in treated male rats (54). Another study showed that administration of mestranol plus norethynodrel induced an increased incidence of AHF in female rats (54). Estrogen-progesteron oral contraceptives carcinogenicity may be mediated by promotion of the epithelium proliferation and by the generation of ROS caused by estrogen reactive metabolites (76).

Human exposure to alpha-particle emitters, in particular Plutonium-239, Radium-224, Thorium-232, has been associated to increased incidence of HCC (57). Recently a 17 month-long study on mice showed that a short period of exposure to Thorotrast (Thorium-232 dioxide) increased the incidence of altered hepatic foci in treated mice (77). A mechanistic explanation is that during their decay radioisotopes emit alpha particles which have been shown to induce direct and indirect DNA damage (78).

Some cases of HCC following tamoxifen treatment in breast cancer patients have been reported (58, 68). Even though no strong statistical association was shown with HCC, clear carcinogenic effects were shown for endometrial cancers. Interestingly, in a 2-year carcinogenicity bioassay, oral administration of tamoxifen to male and female Alderley Park Wistar derived- rats induced an increased incidence of liver adenomas and carcinomas in both sexes (79). Tamoxifen possesses a partial estrogen-agonist activity in the liver (58). Human hepatic estrogen receptor (ER) appears to be quantitative and qualitative similar to that of rodents, so tamoxifen-induced liver tumours in humans and rodents may be due in part to ER-dependent responses, such as hepatocytes mitogenesis (80).

VCM induces increased incidences of hepatic angiosarcomas and hepatocellular carcinomas in humans, as shown in many studies (62, 81-83). However, the first evidence of hepatic angiosarcoma induced by VCM comes from a long-term carcinogenicity study performed in the early 1970s (84, 85) and further experiments showed that VCM induced also HCC and other cancers in rats (85). VCM is predominantly oxidized by the cytochrome P4502E1 and, following metabolic activation, the two metabolites (chloroethylene oxide and chloroacetaldehyde) react with nucleic acid bases to form adducts (62). Furthermore, the molecular pattern of p53 mutations has been shown to be similar in Sprague-Dawley rat primary liver tumours (both angiosarcomas and hepatocellular carcinomas) compared to human liver cancers (86).

Ecological studies performed in areas where drinking water was contaminated by various levels of arsenic (As) have shown increased incidence of different kind of malignancy in exposed population, particularly tumours of skin, lung, liver, kidney and urinary bladder (87-89). In a transplacetal study on CH3 mice, inorganic As was shown to induce a dose-related increase in the incidence of HCC in males (66). Arsenic is genotoxic and induces chromosomal aberrations both *in vitro* and *in vivo (*55, 65).

Interestingly, some chemical agents associated with increased risk for HCC have been reported to be carcinogenic also for the biliary tract in humans. In an epidemiological study on chemical workers vinyl chloride was associated with increased risk of CC (90). Thorotrast, used as a radiocontrast agent in 1930s, has been reported to be a potent carcinogenic agent for CC (91). Furthermore, dioxins and nitrosamines have been associated to increased risk for CC in humans (92). Importantly 3 different kinds of dioxins (2,3,7,8- tetrachlorodibenzo-p-dioxin; 3,3´,4,4´,5-pentachlorobiphenyl and 2,3,4,7,8-pentachlorodibenzofuran) have been recently demonstrated to induce a dose-related increase in both HCC and CC incidence in Sprague-Dawley rats in a 2-year carcinogenicity assay (61).

Many genetic epidemiologic studies have been conducted to evaluate gene polymorphisms and gene-environment interactions as risk factors for HCC, particularly concerning genes coding for metabolizing enzymes (glutathione-S-transferase, epoxide hydrolase, cytochrome P4502E1) or DNA repairing enzymes (XRCC1, UDP-glucuronosyltransferase1A7) (93-98). Until now, results from these studies have reported positive association, association in a subset of population or negative association. These results have been attributed to inadequate sample size to reliably detect likely small effects of single genes on risk with a background of strong environmental factors (5). Meta-analysis and additional studies with larger samples should clarify the rôle of genetic polymorphisms in the onset of HCC (5, 99).

These data clearly indicate that the human liver is sensitive to chemical-induced carcinogenesis and that xenobiotic exposure could play an underestimated rôle in inducing primary liver cancers, alone or in addition to viral and alcoholic etiologies. Although it is aknowledged that in some rodents, particularly in mice, the liver is a preferred target organ for chemical carcinogens (100, 101), it should not be disregarded that some human hepatocarcinogens such as aflatoxins, VCM, oral contraceptives, tamoxifen have been anticipated as such in rodents and that molecular events in tumorigenesis appear to be similar. Even though mouse liver susceptibility to tumorigenesis is often used as an argument against the relevance of carcinogenic bioassays for public health protection, this biological feature should be considered as a *de facto* sensitivite biosensor, as about one third of human carcinogens (IARC group 1) induce HCC in mice (102). Finally, only a small fraction of the about 100,000 chemical agents presently commercialized in Europe and United States have been adequately investigated for their carcinogenic potential, so the chemical burden for hepatocarcinogenesis could be greater than actually estimated (103, 104).

Long-term carcinogenicity bioassays and detection of potential human hepatocarcinogens

Long-term carcinogenicity bioassays, particularly using rats and mice, have been traditionally employed for detecting the carcinogenic potential of chemical and physical agents by the two most extensive bioassays programs in the world, the National Toxicology Program and the Ramazzini Institute (105-107). Although the etiology of hepatic neoplasias is different, in particular due to the absence of chronic hepatitis as a precursor in spontaneous HCC in rodents, histologic, cytological and molecular similarities have been identified between humans and rodents with respect to liver neoplasia (107).

In both humans and rodents HCC are frequently multifocal and show a histologic/cytological spectrum from well-differentiated to poorly-differentiated carcinomas (101). Histological patterns of HCC show various similarities between humans and rodents, as they may be organized in multicellular trabeculae, solid mass or gland-like arrangements (108-111). Furthermore, some histochemical abnormalities are shared between rats and humans, such as decreased activity of ATPase and glucose 6-phosphatase and increased activity of gamma-glutamyltranspeptidase (112).

One of the suggested pathogenic pathways involved in development of HCC is the proliferation of oval cells, considered the stem cells of the adult liver (113). Oval cell proliferation may lead to the formation of atypic hepatic foci (AHF), whose progression towards hepatic neoplasias has been extensively studied in rodents (114-116). AHF has also been indicated as a preneoplastic lesion associated with human HCC development (117).

The molecular pathways involved in the development of liver neoplasia are diverse in both humans and rodents and no universal molecular features have been found associated with all hepatic tumours in both species. In humans, alterations in mainly four different genetic pathways have been identified: the p53 pathway involved in response to DNA damage; the retinoblastoma (Rb) pathway involved in cell cycle control; the TGF-β pathway involved in growth inhibition; and the Wnt/ β-catenin involved in signal transduction and cell-cell adhesion (23, 118, 119). Specific p53 mutations, particularly GC→TA transversions, have been shown after aflatoxin and VCM exposure both in humans and rodents HCC, although p53 mutations in HCC are less frequent in rodents than in humans (86, 120). Other mutations in the p53 gene have been identified in humans, but its overall mutation rate is geographically heterogeneous, ranging from 15% in European HCC to 40% in Chinese HCC (121, 122). It is worth noting that hepatoblastomas have been reported to carry p53 alterations in both children and mice, although in rodents this is a tumour occurring in adult animals (123-125). The Rb gene, an oncosuppressor gene involved in regulation of the G1 phase of the cell cycle and its inactivation, exhibits either gene mutation or epigenetic alterations in about 30% of human HCC (23, 126). Epigenetic silencing through promoter methylation is also observed in another Rb pathway gene, p16-INK4, leading to the repression of cyclin D-dependent kinase inhibitor 2 (CDKN2) and ARF (two tumour

suppressor proteins), in a wide range of HCC (127). In a folate/methyl deficiency model, inducing HCC in rats, methylation of p16 gene promoter has been found to be an early event in hepatocarcinogenesis, suggesting that the Rb pathway is shared by human and rodents in certain conditions and that folate/methyl deficiency model is a suitable experimental model to study Rb pathway implications in hepatic multi-stage carcinogenesis, particularly linked to alterations in DNA methylation (128). Transforming growth factor β (TGF-β), a peptide hormone which controls proliferation and induces apoptosis in hepatocytes, may also play a rôle in early events during hepatocarcinogenesis in both humans and rodents (101, 129). Expression of a co-receptor required for its activation, the mannose-6-phosphate/insuline-like growth factor 2 (M6P/IGF2R), has been found decreased in human and rodent HCC (130, 131). Interestingly, in mice and rats, the M6P/IGF2R gene is imprinted, meaning that just one copy is functional. This fact may partially explain the relatively higher susceptibility of rodents for chemically-induced liver cancers (107, 131). β-catenin is a protein with a dual function in cell-cell adhesion and in Wnt signaling that induces cell proliferation. Point mutations leading to the loss of phosphorylation sites of β-catenin alter its cellular degradation, with consequently protein translocation to the nucleus and transcription of genes, including c-myc, whose upregulation leads to cell proliferation (132). β-catenin mutations seems to be an early event, as they have been found in liver adenomas and in patients with non-invasive hepatocellular carcinoma (133, 134). β-catenin mutations have been also found in chemically-induced mouse HCC, with immunohistochemical accumulation in premalignant liver lesions, suggesting that Wnt signaling pathway is an early event in liver carcinogenesis also in mice alterations and that this pathway may be sensible to chemical carcinogens (135).

CC is a very rare malignancy in rodents. NTP historical controls show CC incidence ranges in Fischer rats from 0-0.1% in males and from 0-0.2% in females, while in B6C3F1 mice it ranges from 0-0.7% in males and from 0-2% in females (136). However, rodent biliary epithelium seems to be sensible to chemical-induced carcinogenesis, as shown by recent long-term carcinogenicity studies on dioxins (61). Moreover an experimental model of thioacetamide-induced ICC in Sprague-Dawley rats has been recently developed (137, 138). A pathological and immunohistochemical study provided evidence that thioacetamide induces the entire range of cholangiocarcinogenesis lesions, from dysplasia to advanced cancerous stage and expression of Epidermal Growth Factor Receptor (EGFR), apomucins and matrix metalloproteinases is similar in human and rat ICC (139).

Although different in etiology, taken together there are a number of similarities in both hystologic aspects and cellular and molecular pathogenesis between humans and rodents concerning the development of primary liver neoplasias. Given that the use of long-term bioassays is a well-established - and currently the most predictive - tool to detect chemical and physical carcinogens, it represents the best experimental surrogate available for detecting potential human hepatocarcinogens.

Animal models for development of novel liver cancer biomarkers

HCC screening is proposed for high risk populations and consists in hepatic ultrasound scanning coupled with serum α-fetoprotein (AFP) assay (140). The use of biological markers for early detection of HCC is currently problematic due to the relatively poor sensitivity of available markers (119, 141). In particular, AFP accounts for a sensitivity ranging from 39% to 64% and a specificity ranging from 76% to 91% (142-143). Moreover, AFP correlates with tumour size and 80% of HCC with tumour diameter < 3 cm do not show increase in AFP serum levels (119, 140, 144). Various serum markers have been proposed to improve HCC detection, such as *Lens culinaris* agglutinin-reactive AFP (AFP-L3); des-gamma carboxyprothrombin (DCP), α-l-fucosidase (AFU), glycopican 3 (GPC3), squamous cell carcinoma antigen (SCCA) and Golgi protein 73 (GP73) (141). None of these proteins has shown enough sensitivity nor specificity to be proposed as unique biomarker to detect early HCC. Many efforts have been dedicated and some improvements obtained by combining two or more of these proteins (145-149), but currently there is not enough data on

their diagnostic accuracy to allow for clinical application, particularly for early detection of HCC. However, it is of note that recently a set of six different serum biomarkers adjusted for age and sex (α-2 microglobulin, haptoglobulin, γ-glutamyl transpeptidase, total bilirubin, apolipoprotein A1 and alanine transferase) has been developed and tested for non-invasive evaluation of liver fibrosis and necrosis in patients with HVC infection, with inter-center reproducibility and with an area under receiver operating curve (AUROC) of 0.87 (150-152). Recently, the association of this set of biomarkers with an ultrasound-based transient elastography has been shown to allow diagnosis and reliable grading of liver fibrosis, avoiding liver biopsy in a significant percentage of patients (153, 154). The same group proposing the panel of biomarkers for evaluating liver fibrosis and necrosis has combined this panel of biomarkers with serum glucose, triglycerides and cholesterol levels and body mass index to evaluate steatosis, obtaining AUROCs ranging from 0.79 to 0.86 (155), even though a comparison with Magnetic Resonance Imaging has shown the latter still being the best method for highly accurate non invasive measurement of liver steatosis (156).

Recent advances in global genomic and proteomic approaches could help improve early diagnostic of primary liver cancers. DNA microarrays allow simultaneous analysis of thousands of genes and will probably amplify the discovery of new markers, but standardization and interpretation difficulties are currently limiting the use of these methods in clinical practice. Serum proteomics using surface-enhanced laser desorption/ionization-time of flight mass spectrometry (SELDI-TOF MS) has been recently used to develop new HCC biomarkers (157). A study comparing sensitivity and specificity of SELDI-TOF MS with AFP, AFP-L3 and DCP showed higher sensitivity and specificity for the proteomic analysis (158). There are different strategies for the search of protein biomarkers, including analysis of pathologic tissue, identification of tumour antigens that induce autoantibodies directed against HCC antigens, but the most interesting for potential early diagnosis is analysis of serum of other biological fluids. Beretta reports that although many technical advances have been achieved, some basic questions remain to be answered, particularly the lack of information on differences in plasma proteome in healthy subjects differring for sex and age and the specific design of studies aimed to detect early neoplastic lesions which cannot be diagnosed with available tools (159).

In this context, the use of experimental models of HCC and CC could provide a valuable platform for development and validation of new biomarkers. In particular, the collection of biological fluids is feasable throughout the life of animals, before and after they may develop a neoplasia during long-term carcinogenicity bioassays, notably life-span experiments (106, 160). This approach combines the possibility of both prospective and retrospective analysis of plasma and urine, allowing the identification of promising biomarkers for early detection of cancer. Furthermore, life-span carcinogenicity bioassays provide access to a larger number of samples (at least 100 animals/group) compared to clinical studies, thus reducing the effect of chance in detecting a potential candidate molecule (161). The human homologues of the most predictive biomarkers identified experimentally could be tested in clinical and population studies, possibly reducing costs and time of clinical research and increasing successful identification of useful biomarkers. In recent years, *in vivo* imaging methods, such as ultrasound, Magnetic Resonance Imaging (MRI), Computed Tomography (CT) and Positron Emission Tomography (PET), have improved, allowing high spatial resolutions, informative metabolic, functional and molecular information also in laboratory animals (162, 163). Use of these imagining methods is rapidly growing in physiopathological and drug discovery studies in preclinical models. Furthermore, algorithms have been developed that superimpose information obtained from different imaging devices such as CT, PET or MRI, allowing additional spatial and quantitative data (164). Well-designed protocols which will integrate proteomic approach with imaging diagnostics using animal models of cancer may result in greater improvement for biomarkers development.

Many experimental models of preneoplastic and neoplastic liver lesions are already available in rodents. Liver fibrosis and cirrhosis can be induced in rats by treating them with carbon tetrachloride or phenobarbital or a combination of both, reproducing

the development of cirrhosis in human livers (165, 166). These models are currently used in testing new potential antifibrotic agents and they could provide a valuable model for early detection biomarkers research since rats, like humans, may develop HCC in cirrhotic livers (167). Diethylnitrosamine (DEN) also induces HCC in exposed rats and provides a widely used model to study mechanisms of hepatocellular carcinogenesis (168-170), so it has potential to be exploited for biomarkers development. Rodent models of NASH, recently proposed as a risk factor for HCC (47), are also available. These include genetic models and diet-induced NASH. Genetic models such as ob/ob mice (leptin-deficient) or phosphatase and tensin homolog (PTEN)-deficient mice, have been shown to be more prone to develop HCC, thus renforcing the hypothesis that NASH can represent a precancerous lesion in the liver (171, 172). Diet-induced NASH, like in the case of methionine-choline deficient feeding in both rats and mice, are providing valuable results in understanding biochemical and molecular pathological processes underlying onset and progression of NASH (173, 174).

As previously described, a chemical-induced model of CC treating rats with thioacetamide is also available. Although the capacity of thioacetamide to induce CC has been known since the mid-1980s (175), its use to study mechanisms of CC tumorigenesis and drug discovery is quite recent (137, 176). This model opens new possibilities to explore markers for CC, for which early detection could improve clinical and surgical management of the disease and possibly survival.

Conclusions

Primary liver cancers, particularly HCC and CC, are an increasing public health problem in both developed and developing countries (1, 2). Although viral and alcoholic etiologies seem to account for most HCC, recent evidence show that an increasing fraction of HCC arise in people having no recognized risk factors (5, 49). Furthermore, the recent increase in the incidence of CC is likely to be of environmental origin, as other known risk factors do not appear to have increased in the last decade (6). Long-term carcinogenicity bioassays on rodents are the best available tool to study the carcinogenic potential of chemical and physical agents and provide a unique resource to improve primary prevention of cancer in general and also of liver cancers (106, 107, 160). The fact that some rodents, particularly mice, have a higher susceptibility for developping HCC should not be considered as a shortcoming in long-term carcinogenicity studies, but rather as an available sensitive biosensor for detecting carcinogenic potential. Furthermore, animal models are now available to develop and validate early detection or exposure biomarkers for liver neoplasia or hepatocarcinogens. More research and well-designed protocols are needed to integrate long-term carcinogenicity bioassays and exposure/early detection biomarkers research in order to decrease the global burden of cancer incidence and mortality.

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