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Role of mTOR system and its relation with somatostatin and dopamine receptors on cell proliferation, hormone secretion and intracellular signalling in hepatocellular and cholangiocellular carcinomas.

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Abstract

Hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC) are among the most lethal of human malignancies with s a very poor prognosis, with a <3% 5-years survival rate for untreated cancer. The actual therapies are limited to a little percentage of patients they are often diagnosed in advanced stages or even in metastatic conditions. In the last years, the idea of an "omics revolution" has given the basis to a new kind of therapies cancer, a molecular approach. In this work, we have focused our attention on mTOR pathway, its inhibitors and on their antiproliferative effects evaluating not only cell lines viability decrease after treatment with them, but also the molecular mechanism at basis of this phenomenon. In addition, our research project has investigated also somatostatin and dopamine receptor expression with the purpose to identify a molecular profile of this cancer, using four different cell lines and 23 Caucasian patients. So, stimulation of these receptors with the correspondent ligands has demostrated that their best therapeutical effect is observed with use of drugs with affinity for two different receptors, probably due to heterodimerization of somatostatin receptors. These data have been confirmed by different kinds of experiments (evaluating proliferation, hormone secretion and intracellular signalling) revealing an emergent aspect of HCC, its neuroendocrine fenotype. Our future purpose is to clarify better some molecular aspects that regulate heterodimerization and to define the intracellular pathways activated by these analogues. We, also are going to evaluate the effects of a potential combination between mTOR inhibitors and somatostatin analogues underlining a possible signalling interaction.

Introduction

Hepatocarcinoma (HCC) and cholangiocarcinoma (CC) rapresent respectively the first and the second malignancy most frequent among the primary liver tumors. HCC and CC have a very poor prognosis, with a life expectancy of about 6 months from time of diagnosis and a <3% 5-years survival rate for untreated cancer. The actual therapies are limited to a little percentage of patients because HCC and CC are often diagnosed in advanced stages or even in metastatic conditions. In the last years, the idea of an "omics revolution" has given the basis to a new kind of therapies cancer, a molecular approach. For HCC, in particular, there have been identified several altered genes involved in what is defined, "the multistep hepatocarcinogenesis". The documented ability of these neoplasms to acquire a neuroendocrine phenotype during the clinical course has prompted the study of endocrine patterns as potential targets for the treatment of these tumors usually chemoresistant. Several studies in the literature report the expression of somatostatin receptors (SS) and dopamine (D) in neuroendocrine tumors (NETs). Currently, however, the role of somatostatin receptors in HCC and CC is still controversial while dopaminergic system is completely unknown in HCC.

Activation of the SS and D receptors means the start of signalling process through different intracellular pathways including PI3K/Akt. This pathway in turn activates the protein known as mammalian target of rapamicyn (mTOR), which is hyper-expressed and deregulated in NETs. All these considerations suggest that the SS, D and m-TOR system may have a crucial role in the care of patients with HCC and CC.

Given these assumptions, this work aims to investigate what are the pathways activated by somatostatin and dopamine receptors and their relationships with mTOR.

Liver tumors

Liver anatomy

The liver is the most voluminous gland of the human body located in right hypochondrium. The four main cell types that are in the liver are hepatocytes, stellate cells (HSCs), sinusoidal endothelial cells and Kupffer cells.

The **hepatocytes** are the most numerous cells of the liver, they constitute 80% of the volume. Their shape is multifaceted and their diameter varies from 20 to 30 microns. They are often tetraploid and multinucleated, with a number of cores that can be up to four, a large nucleolus, well developed smooth and rough endoplasmic reticulum, numerous Golgi cisternae, ribosomes, lysosomes, mitochondria and peroxisomes. They represent one of the cell types in which the organelles are more developed and presented, due to the high metabolic needs and the wide variety of tasks they must perform. In a well-nourished body is not difficult to detect moderate amounts of glycogen and lipid vacuoles, or, in case of overdose of iron, vacuoles or aggregates of ferritin and hemosiderin. The eosinophilic cytoplasm is due to the large number of mitochondria but with numerous basophilic granulations due to the rough endoplasmic reticulum and ribosomes. There are also present golden-brown lipofuscin granules.

Stellate cells (**HSCs**) or Ito cells, of mesenchymal origin and much less numerous than hepatocytes, are placed at the base of the hepatocytes, and have a star-shaped or irregular shape. Their cytoplasm is rich in vitamin A-containing lipid vesicles, and their job is to secrete the main matrix constituents, including type III collagen and reticulin. They are essential in liver regeneration following injury or surgery, as they secrete growth factors responsible for the good ability to regenerate the liver. In case of injury can replace damaged hepatocytes by the secretion of collagen and other structural proteins, forming scar tissue.

The **sinusoidal endothelial cells** form the endothelium of fenestrated venous sinusoids of the liver. They have flat shape, oval nucleus centrally located, scant cytoplasm containing numerous vesicles trans-cytotic, all them are united by joints. The fenestrations exist between the cells are very large and combined in complex with an average diameter of 100 microns, so that blood can easily be poured into Disse spaces and come into contact with the hepatocyte microvills.

Kupffer cells, liver macrophages, derived from monocytes and take placed in the lumen of the venous sinusoids. Their form is variable and irregular, with numerous protrusions typical of cells of the macrophage that extend into the lumen of the sinusoid. Their function is to remove any debris by phagocytosis in the blood flow to hepatocytes, but may also stimulate the immune system through the secretion of numerous factors and cytokines. They remove old or damaged red blood cells acting as a complement to the spleen (which can be substituted in case of splenectomy).

The internal structure is lobular, the parenchyma is in fact composed of a large number of elementary units, the lobules, with all the same and independent function. In the interlobular spaces, also known as portals, starting in the last ramifications of the portal vein, through which the blood reaches the lobule from the gut, laden with substances absorbed during digestion. In the axis of the lobule the centrilobular vein runs, this is the source of so-called circle of the liver or portal circulation, that of the hepatic veins tributaries of the inferior vena cava. Between the vein and the periphery of the lobule the centrilobular liver cells are arranged in columns arranged radially, leaving between them spaces (sinusoids) through which the blood from interlobular veins reaches the centrilobular vein (Fig.1.1). Blood flows in direct contact with the sinusoids in liver cells because it lacks a true endothelium. There are only cells, called Kupffer, whose function is related to the synthesis of bilirubin from hemoglobin. In the columns of liver cells as well starting in the lobule bile capillaries, which converge at the periphery of the lobule, giving

life to the bile ducts. These, in turn, at the hilum of the liver, merge into a single duct (bile duct),

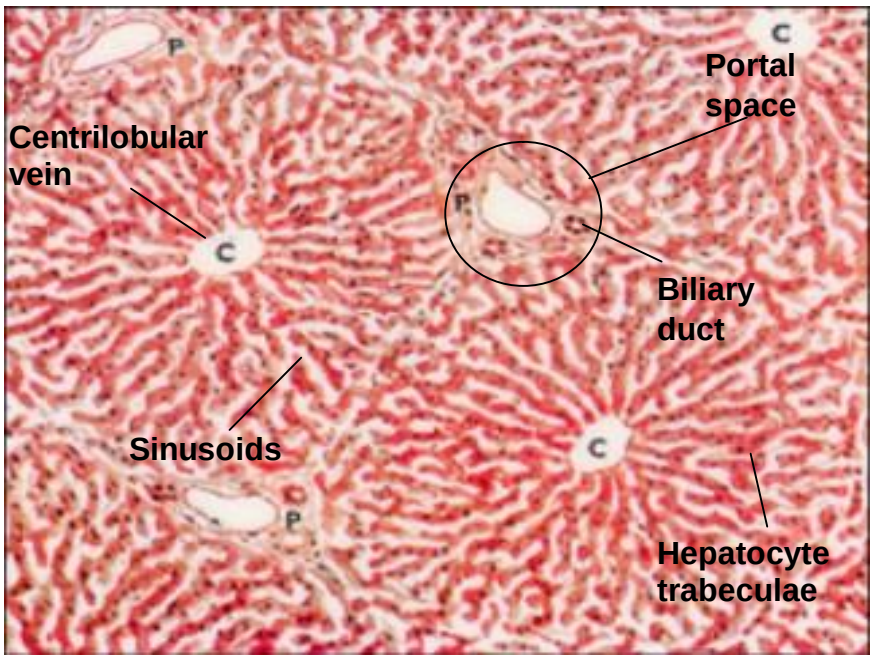


Fig.1.1: liver anatomy.

which with the cystic duct, coming from the gallbladder, is to form the common bile duct carries bile to the duodenum. In addition to the blood-vessels, lymphatic vessels leave the liver, divided into deep, which originate from the lobules, and surface, forming a network under the serous membrane. The nerves of the liver are derived from the celiac plexus and the vagus and form along the hepatic artery and its branches, the hepatic plexus.

Liver physiology

The various functions of the liver are carried out by the liver cells or hepatocytes. Currently, there is no artificial organ or device capable of emulating all the functions of the liver. Only some functions can be emulated by liver dialysis, an experimental treatment for liver failure. Over all the functions of which liver is responsible, the most important are the aminoacids synthesis; carbohydrate metabolism (Gluconeogenesis, Glycogenolysis, Glycogenesis); protein synthesis as well as degradation; lipidic metabolism with cholesterol

synthesis and lipogenesis, the biochemistry process that products triglycerides. Furthermore, the liver produces coagulation factors I (fibrinogen), II (prothrombin), V, VII, IX, X and XI, as well as protein C, protein S and antithrombin; moreover, albumin and angiotensinogen. In the first trimester fetus, the liver is the main site of red blood cell production. By the 32nd week of gestation, the bone marrow has almost completely taken over that task. The liver produces and excretes bile (a yellowish liquid) required for emulsifying fats. Some of the bile drains directly into the duodenum, and some is stored in the gallbladder. The liver also produces insulin-like growth factor 1 (IGF-1), a polypeptide protein hormone that plays an important role in childhood growth and continues to have anabolic effects in adults. The liver is a major site of thrombopoietin production and of storage of different substances, such as, glucose in the form of glycogen, vitamins A, D and B12, iron and copper. Additionally, the liver is responsible of immunological effects because its reticular endothelial system contains many immunologically active cells.

Liver diseases

Primary malignant liver tumors

Even liver tumors, as well as for each organ can be divided into benign and malignant. Malignant tumors can in turn be primitive (born from the cells of the liver) or secondary (metastases of primary tumors of other organs). The primary malignant liver tumors including hepatocellular carcinoma, the cancer far more frequently, cholangiocarcinoma, and angiosarcoma, and represent, worldwide, a leading cause of cancer death [1].

Hepatocarcinoma (HCC)

Hepatocellular carcinoma is the third leading cause of death in the world due to cancer-related death with approximately 500000 deaths every year [2] despite having a different

impact depending on the region considered. In Italy is a health issue of great importance, due to the high prevalence of viral hepatitis

HCC tumorigenesis and risk factors

Hepatocellular carcinoma is the result of different genetic and epigenetic alterations that accumulate during the various stages of carcinogenesis [3] (Fig. 2.1). In fact, the hepatocarcinogenesis is a multistep process that involves profound alterations in the cellular genome and in which we can identify at least three main stages: induction, promotion and progression [4] [5]. In the first stage (induction), malignant transformation is caused by genetic mutations dell'epatocita induced by a chemical agent beginning (eg aflatoxins, drugs) or viral (hepatitis B virus, HBV) or secondary to an increased cell turnover . These mutations lead to altered expression of some proto-oncogenes (ras, myc, fos) and consequently a dysregulation of cell cycle [6]. These cellular changes are irreversible, but the cells involved may be removed by the liver via apoptosis. The second stage (promotion) is the proliferation of transformed cells, which requires the presence of a continuous or repeated stimulation, such as the persistence of the damage necro-inflammatory chronic (chronic hepatitis) associated with regeneration (cirrhosis) [7]. The promotion of cancer is countered by the interference of sex hormones, cytokines, enzymes (eg the ornitindecarbossilasi, ODC) or cyclins (involved in cell proliferation). The progression of carcinogenesis to clinical manifestation of cancer is due to clonal expansion of cancer cells, mediated by growth factors (IGF-2, TGF-α, TGF-β) and facilitated by additional genetic mutations involving tumor suppressor genes such as p53 [8] [9]. So exposure to "risk factors", as well as toxic substances can initiate immune responses to the necrotic process and stimulate inflammation through activation of Kupffer cells and HSC. The HSC acquire a myofibroblast phenotype, and this process to guide the development of fibrosis through excessive release of extracellular matrix (ECM). This deposit of ECM often

results in a reduction of blood flow in the liver. Fibrosis can develop into cirrhosis which is one of the most important risk factors for the development of HCC [1]. Cirrhosis involves a transformation in the architecture of liver lobules, with the formation of nodules that affect the organization and function impairment. In more than 80% of cases, HCC is associated with cirrhosis, which in turn is mainly due to chronic viral infection (HBV or HCV) or alcohol abuse [1]. The major risk factors for HCC are shown in *Table 1.1.*

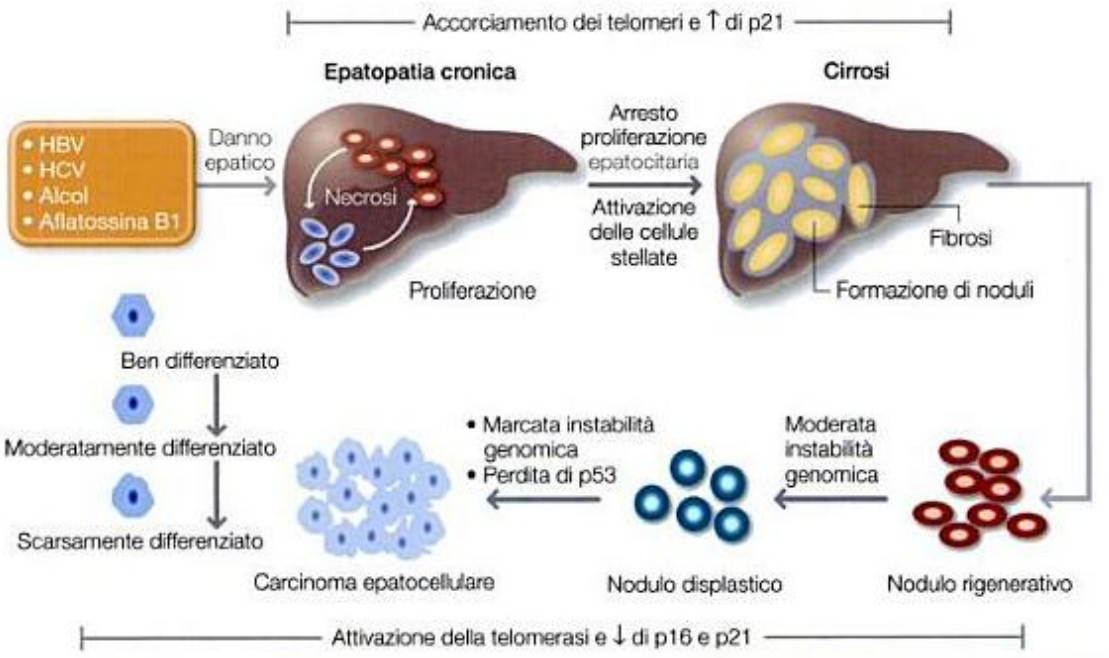


Fig 2.1: histopathological progression and molecular characteristics of hepatocarcinogenesis. Different etiological factors causing liver damage characterized mainly by necrosis and hepatocyte proliferation. An accelerated telomere shortening in the course of chronic liver disease with cirrhosis induces the accumulation of senescent hepatocytes in cirrhotic stage so they are particularly sensitive to undergo molecular alterations predisposing to the development of neoplasia, such as chromosomal instability. Only in the final stage of hepatocarcinogenesis there is a reactivation of telomerase (from M. Colombo 2010)

80%	Cirrhosis or advanced fibrosis, mainly due to: • Hepatitis B virus infection • Hepatitis C virus infection • Alcohol • Non-alcoholic steatohepatitis (NASH) • Congenital disorders such as hemochromatosis, Wilson's disease, etc. ...
20%	In absence of cirrhosis or advanced fibrosis: • Hepatitis B virus infection • Aflatoxins (often associated with HBV infection) • Genetic pathologies • Pharmacological induction (eg anabolic steroids)

Table 1.1: Main risk factors for HCC.

Hepatitis viruses

Hepatitis B virus (HBV) and C (HCV) infections are the most important risk factors, being involved in more than 70% of HCC cases worldwide [10]. In adults, HBV infection is greater in acute hepatitis and in 10% of these infections develop chronic hepatitis that can lead to development of cirrhosis and eventually HCC [1]. HBV belongs to the *Hepadnaviridae* (Fig. 3.1). This virus has a genome to double-stranded circular DNA partially coding for 4 proteins completely different: the surface proteins (S, PreS1, PreS2), expressed on the surface of the virion infectious or released in the serum of the host with power highly immunogenic; Core protein has a structural function but plays an important role in the life cycle of the virus DNA polymerase activity with polymerization, reverse transcription and ribonuclease activity, and finally the protein trans-activating capacity X and can over-regulate the activity the HBV genome. In addition, it influences the expression of a several number of genes including those involved in host survival, cell cycle progression and invasion [11] [12] [13].

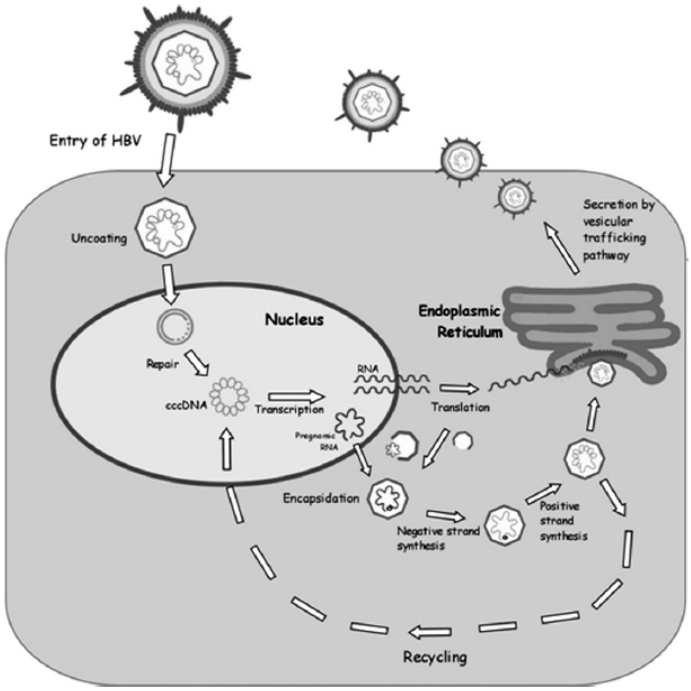


Fig.3.1: Life cycle of hepatitis B virus.

The "*integration"* of HBV-DNA into the chromosomal DNA, which occurs in discrete sites and variables, can cause deletions, chromosomal re-arrangements or alterations of genes involved in regulating cell proliferation and differentiation, viral integration may also occur in at or near protooncogenes [14]. HBV-DNA also contains sequences that encode proteins capable of *transactivation* cellular genes: sequences of the regions pre-S / S and truncated region X. The genes (open reading frame, ORF) for proteins with transactivation activities often are kept intact in mini-chromosomes (HBV-DNA supercoiled, ccc-DNA) in hepatocytes of patients with previous HBV infection. Finally, HBV-DNA sequences were detected in HCC patients negative for all serological markers of HBV infection, suggesting a role of occult HBV infection in the pathogenesis of HCC in these patients [15] (Fig. 4.1).

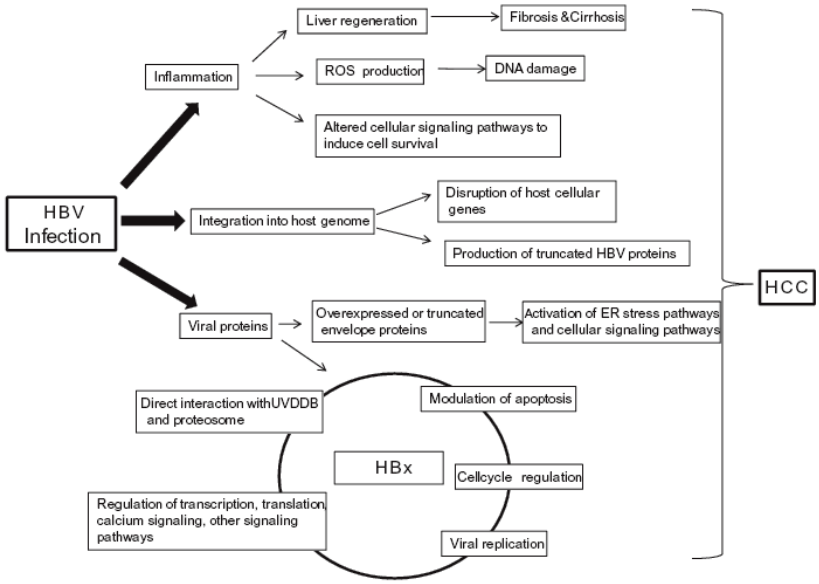


Fig.4.1: hepatocarcinogenesis induced by HBV.

HCV belongs to the family *Flaviviridae*, has a pericapsid and a single-stranded RNA genome with positive polarity: 5'-3 '. It completes its life cycle in the cytoplasm (Fig. 5.1) and in the absence of an enzyme activity of reverse transcriptase can not integrate into the host genome, so a mechanism of insertional mutagenesis can be excluded for the development of HCC associated with chronic infection HCV [10]. Its genome encodes a single polyprotein of 3000 amino acids that is processed by post-transcriptional viral and cellular proteases into 4 structural proteins (core, E1, E2 and p7) and 6 non-structural

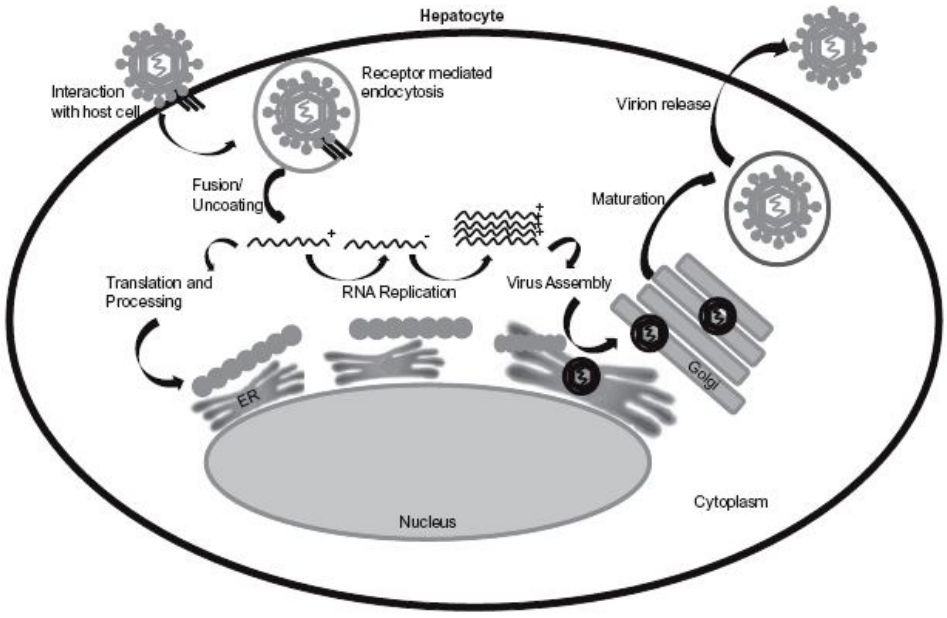


Fig. 5.1: Life cycle of hepatitis C virus

proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B).

The translational process of viral proteins occurs in the endoplasmic reticulum of host cells. The non-structural proteins are essential for the replication of viral RNA. The assembly of the RNA and of the external structure occurs in the Golgi. HCV has been detected in various forms in heterologous blood of infected patients. In fact, the virus is present in free form, which is associated with lipoprotein at high, low and very low density or immunoglobulin [16]. Hepatocytes are the main target of HCV, but infections have also been reported in other cell types such as B cells and dendritic cells [17]. HCV infected hepatocytes (Fig. 5.1), but does not kill the cells, rather triggers an inflammatory response immune-mediated (hepatitis) that is sometimes due to the development of HCC. Attack of the flavivirus cell surface generally causes endocytosis of bound virions. Some receptors including CD81, the surface protein of group tetraspanine, the LDL receptor (LDLR) and claudin-1, mediate adhesion and trafficking of this family of viruses in endocytosis vesicle [16] [17]. Cirrhosis appears to be a pre-requisite in the development of HCC HCV-induced. The viral proteins lead to cell transformation indirectly by regulating the proliferation and cell cycle control through various mechanisms including the binding and the modulation of several tumor-suppressor proteins including p53, p73 and Rb. Several intracellular signalling have been shown to have a role in HCV-induced hepatocarcinogenesis, including oxidative stress, stress of the endoplasmic reticulum, Ca^{2+} signaling, TGF-ß, p53, Raf / MAPK and Wnt / ß-catenin.

Cirrhosis

Cirrhosis is a chronic disease very common in Italy. It is one of the main risk factors for HCC and it is associated to hepatocarcinoma in approximately 90% of cases (at least in Italy and in western countries). It is an affection of the liver characterized by the replacement of normal liver tissue with connective tissue (sclerosis) that alters the liver structure and functionality. As a result of chronic inflammatory stimuli and actions of the

so-called cirrhogenic factors (leading to cirrhosis), liver tissue is damaged ranging from degeneration (steatosis more frequently) to necrosis. The cell death stimulates the proliferation of fibroblasts present in the liver, which produce large quantities of connective tissue collagen, the characteristic tissue of healing. But probably the fibroblasts are stimulated even before by the same cirrhogenic factors that cause cell death. So the lobules, which are the architectural element of the liver, are surrounded and as "strangled" by these newly formed connective branches. Thus, there is parallel to cell death and a further proliferation of fibroblasts: accordingly, the hepatocytes starts to proliferate in order to repair the damage, giving rise to new lobules, also known as pseudolobuli which however are very different from normal lobules, with architecture subverted and deprived of the usual vascular connections. Another typical manifestation of cirrhosis is the ascites that is the accumulation of a protein-poor fluid (transudate) in the peritoneal cavity, which confers a globular shape, very large, to the abdomen and has the characteristic to reform it when the liquid is extracted with paracentesis. In ascites, complex mechanisms come into play, first the portal hypertension causes a leakage of fluid from the capillaries of the portal vein, and especially from those located at the peritoneum and at the liver capsule. Moreover, as a result of impaired functionality of the organ, proteins (albumin and globulins) are not synthesized, also there is a decrease of plasma proteins (hypoproteinemia). As one of the functions of these proteins is to draw water from the interstice and to oppose to his release in the capillary, their decline leads inevitably to an increase of liquid amount that accumulates outside the vessels. Cirrhosis can play a dual role in the oncogenetic process both as a causing factor that as a promoting factor. Indeed, the increased cell turnover secondary to chronic necroinflammatory injury and subsequent hepatocyte regeneration may contribute directly to increasing the frequency of spontaneous genetic mutations, and then can select cell clones with characteristics of neoplastic type. Secondly, the stimulation of "started" cell proliferation may promote the progression to the overt malignancy.

Aflatoxins

Worldwide, the principal liver carcinogen present in food is aflatoxin, a product of the metabolism of the fungus *Aspergillus flavus* which contaminates the food (usually the produce of grain stored in hot and humid climates) in many tropical countries, particularly in Southern Africa and South-East Asia. Experimentally, it is among the most potent liver carcinogen known for certain animal species and it is likely that it is also for men. In addition, the incidence of hepatocellular carcinomas in some primitive areas of Africa (where this tumor is particularly popular) is positively correlated with the content of aflatoxin in the diet. [18].

Cholangiocarcinoma (CC)

Cholangiocarcinoma (CC) originates from neoplastic transformation of cholangiocytes (epithelial cells lining the bile ducts) and can develop in different sections of the biliary tree for which the CC can be: intrahepatic, hilar or extrahepatic [19] (Fig. 6.1). In Europe, approximately 50000 new cases of primary liver cancer are diagnosed each year and about 20% of these are attributed to CC [20].

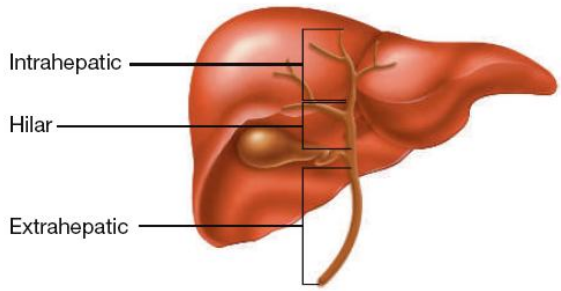


Fig. 6.1: biliary tree and location of the cholangiocarcinoma

Risk factors

Several risk factors, summarized in *Table 2.1*, were associated with the development of CC.

Some are well-recognized risk factors for this cancer, such as congenital anomalies of the bile ducts, primary sclerosing cholangitis (PSC), the hepatolitiasi, parasitic infections, bile duct adenoma, biliary papillomatosis, and genetic risks.

Established risk factors:

- *Opisthorchis viverini* infection
- primary sclerosing cholangitis
- Hepatolitiasi
- Choledochal cystis
- Hepatic cirrhosis
- Hepatitis C virus infection
- Congenital anomalies of the biliary tract

Probable risk factors:

- Hepatitis B virus infection
- *Clonorchis sinesi* infection
- HIV infection
- Diabetes
- Exposure to toxins, dioxin and PVC

Table 2.1: Major risk factors for the development of CC

The cytokines produced by cholangiocytes and macrophages can modulate gene expression and lead to activation of carcinogenic metabolites. For example, interleukin 6 (IL-6) is a potent mitogen for cholangiocytes, since induces the nitric oxide synthase expression (NOS) (Fig. 7.1). Nitric oxide can directly damage DNA [21]. Congenital biliary cysts and pancreatic-bile duct malfunction have been associated with biliary carcinogenesis, both of which are present in 90% of cases reported in several clinical cases. Mixture of bile and pancreatic fluid induces chronic inflammation in the biliary tree with possible activation of the carcinogenic cascade. The PSC is one of the most common causes of CC in the West, although the duration of the PSC is not a risk factor for the development of CC. Furthermore, mutation of the K-ras gene has been associated with PSC.

Parasites such as *Clonorchis sinensis* and *Opisthorchis viverini* have been shown to have direct effects on carcinogenesis by increasing the susceptibility of cholangiocities to endogenous and exogenous carcinogens.

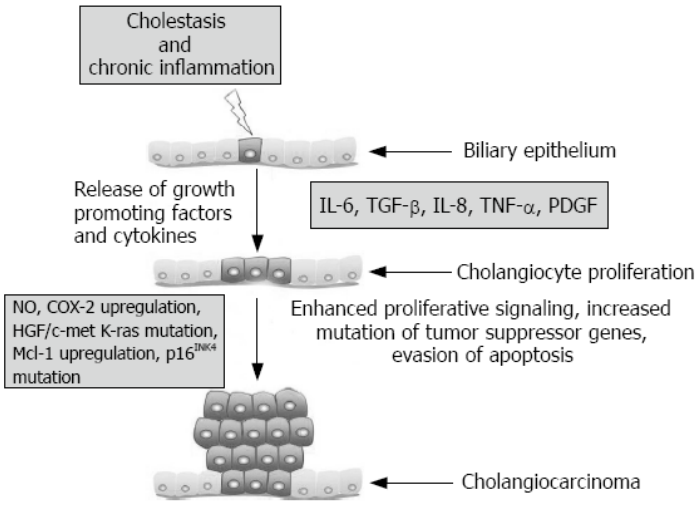


Fig. 7.1: Inflammation and carcinogenesis.

Hepatocellular carcinoma and cholangiocarcinoma exhibit a neuroendocrine phenotype

The term "neuroendocrine" has been used to define the phenotype of cells that secrete their products in a regulated manner, in response to a specific stimulus. The neuroendocrine system includes neurons and endocrine cells phenotypically characterized by the expression of markers such as neuropeptides and chromogranin. Neuroendocrine features have been used as evidence of a common embryological origin for normal and neoplastic cells. However, it is now recognized that neuroendocrine characteristics can be observed in various cell types that do not have a common embryological origin with neurons and endocrine cells. Although hepatocellular carcinoma and cholangiocarcinoma are not commonly classified as neuroendocrine tumors, both typically exhibit neuroendocrine features. Many studies show high serum concentrations of chromogranin (Cg) A in HCC. Furthermore, the increase or decrease in the levels of CgA correlates with the degree of differentiation of HCC [22] [23]. Another feature of neuroendocrine cells of HCC is the expression of somatostatin receptors, G protein-coupled receptors with inhibitory capacity growth, proliferation and secretion in some NETs [24].

Many hormones are involved in the neuroendocrine biology of cholangiocytes, including somatostatin, which stimulates the absorption of bile, serotonin, which reduces cell growth, insulin, gastrin and estrogens, which respectively reduce and stimulate the proliferation cell, the IGF-1 also it stimulates the proliferation, and dopamine [25].

Moreover, in case of cholestasis, cholangiocytes stimulating the proliferation and secretion of several neuroendocrine factors, play a strong phenotype by triggering a neuroendocrine loop autocrine / paracrine regulating the proliferative response to cholestasis [25] (Fig. 8.1).

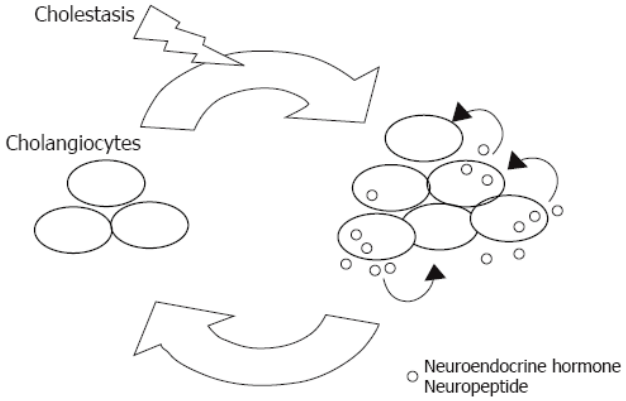


Fig.8.1: Acquisition of neuroendocrine phenotype and trigger autocrine loop by cholangiocytes in cholestasis conditions.

Study of somatostatin, dopamine and mTOR pathways in liver diseases

Somatostatin and cancer

Somatostatin, SST (also known as growth hormone-inhibiting hormone (GHIH) or somatotropin release-inhibiting factor (SRIF)) is a peptide hormone that regulates the endocrine system and affects neurotransmission, cell proliferation and numerous secondary hormones release inhibition. Two active biologically forms derive from the C-terminus portion of a single pro-peptide: SST-14 and SST-28. SST acts on its multiple cell targets via a family of six receptors that originate from five genes: SSTR1, SSTR2a, SSTR2b, SSTR3, SSTR4, SSTR5. SSTR2 is alternatively spliced at its C-terminus producing the SSTR2a and the SSTR2b variants that have a somewhat different tissue distribution. the SSTRs. Besides their expression in normal tissues, SSTRs have been identified in tumor cell of different aetiology including pituitary, pancreatic, breast and hematopoietic tissues. Moreover, the majority of human tumors do express somatostatin receptors, often more than one receptor subtype [26]. As mentioned above, the SST receptors are members of the G- proteins coupled receptors (GPCRs) superfamily and so modulate cellular function through multiple pathways coupled to G-protein dependent signalling pathways. The different signalling pathways activated by the various sstr subtypes vary according to the receptor subtype and tissue localization. However, all sstr subtypes inhibit Adenylate Cyclase and cAMP production upon ligand binding [27]. All of the pleiotropic effects of SST in the different target tissues can be explained by two basic biological mechanisms: inhibition of secretion and inhibition of proliferation. As already mentioned, the SST peptides inhibit secretion (of neurotransmitters or hormones) from cells in different tissues such as the pituitary gland, the endocrine pancreas and the stomach. The molecular mechanism by which SST exerts

its inhibitory effects on cell secretion, is still a matter of intense study and may vary between the different cell types. However, it is generally accepted that after binding its receptors, somatostatin or somatostatin analogues, actives an intracellular trasductional message that downregulates the enzyme Adenilate Cyclase, wich in turn inactives Protein Kinase A (PKA), leading to an the intracellular decrease of both cAMP and Ca^{2+}. As reported by literature this signalling is mainly responsible for the secretion inhibition, with some effects due to the activation of phosphatases such as calcineurin [28]. Another intracellular pathway activated by this neuropeptide, in fact, shows the upregulation of some phosphatases belonging to different families such as serin-threonin kinases (PTPases, SHP-1 and SHP-2) or Ca^{2+} dependent, as for example calcineurin. The anti-proliferative effects of SST are largely believed to be due to the activation of tyrosine phosphatases that dephosphorylate (and inhibit) growth factor receptors. In addition, the SST-mediated activation of phosphatases regulates more distal signalling pathways such as the MAPK pathway. Addition of SST (or synthetic analogues) to sstr expressing proliferating cells usually produces cell growth arrest at the G1 phase of the cell cycle [29]. Interestingly, in some cells, activation of the sstr2 and sstr3 subtypes induced apoptosis and cell death rather than growth arrest through activation and upregulation of the tumor suppressor p53 and the pro-apoptotic protein Bax [30]. In fact, the expression of SSTRs in several human tumors was so pervasive that it helped to create an entire new field in oncology: peptide therapy. SST analogues have also been used in direct tumor reduction with 90Y radiolabeled analogues and in the symptomatic treatment of hormone secreting tumors [31-32]. After agonist activation, GPCRs are phosphorylated (involving protein kinase A, protein kinase C, and GPCR kinases) and internalized, probably via the formation of clathrin-coated pits (involving β-arrestins). The internalized receptors are then directed to endosomes in which they are dephosphorylated. Subsequently, the receptors are recycled back to the plasma membrane as functional (resensitized) receptors. GPCR down-regulation results from lysosomal degradation of intracellular receptors, decreased mRNA

and receptor protein synthesis, as well as increased degradation via mobilization of membrane receptors directly to the lysosomal compartment [33-35]. Actually, the presence and the role of somatostatin receptors in the liver disease is not yet clarified because controversial results on tumor progression and patients survival have been obtained [36-39]. On the other hand, the somatostatin analogue octreotide have been found to inhibit α-fetoprotein (AFP) secretion and cell proliferation in HCC *in vitro* [40]. Moreover, an heterogeneous expression of the somatostatin receptors have been demonstrated by imunohistochemistry in HCC. Indeed, a study on 56 cases of HCC demonstrated an individual differential expression pattern and levels for the different somatostatin receptors, being sst1 and sst2 less frequent and intense than sst3 and sst5 [41]. On the other hand, a study on 6 cases of HCC confirmed that sst1, sst2, sst3 and sst5 are variably expressed in HCC, but sst1 and sst2 were demonstrated to be dominant compared with sst3 and sst5 [42].

Dopamine and cancer

Dopamine, an important member of the catecholamine family, is one of the major neurotransmitters in the mammalian brain and it also has an independent role in the periphery [43]. The various actions of dopamine are mediated by five specific receptors (D1–D5), which can be subdivided in two different receptor families on the basis of their biochemical and pharmacological characteristics: D1-like (including the D1 and D5) and D2-like (including theD2, D3, and D4 receptor). D2 receptor exists in two different isoforms, the long (D2 long) and short (D2 short) isoforms [44]. Dopamine has a significant influence on the immunological defense system of the host either directly or indirectly through the regulation of the secretion of prolactin (PRL) and growth hormone because these hormones in turn have been shown to modulate the immune system [45]. Moreover, reports are also available indicating the direct antiproliferative effect of

dopamine and its analogs on malignant cell proliferation both *in vivo* and *in vitro*. Therefore, the role of dopamine as a potential regulator of malignant cell proliferation, functional activities of hormones, and the immune system in tumor-bearing hosts has been discussed [45]. Moreover, Rocheville et al provided the evidence that receptors from different G protein (heterotrimeric guanine nucleotide binding protein)–coupled receptor families interact through oligomerization and they characterized the interaction between the long form of the human D2R and human SSTR5. They also demonstrated that the hetero-oligomerization of SSTR5 and D2R was induced by ligand binding, that ligand binding to either receptor can trigger hetero-oligomer formation and that there are no preformed hetero-oligomers in the absence of ligand. Hetero-oligomerization led to synergy of the two functional receptor resulting in enhancement of dopaminergic and somatostatinergic transmission induced by in vivo administration of SST or dopamine agonists [46]. Given this discovery, it has been possible to investigate several cellular models expressing both receptor subtypes to understand how these receptors may be involved in inhibiting the proliferation of different cancers. Currently, the presence of dopamine pathway has been investigated only in cholangiocarcinoma by Coufal et al. The authors demonstrated that there was an increase in the expression of the enzymes responsible for dopamine synthesis, which led to an increased accumulation and secretion of dopamine from cholangiocarcinoma; moreover a specific inhibition of dopamine production led to a suppression of tumor growth in a xenograft model of cholangiocarcinoma suggesting an effect of pro-growth of this pathway in this devastating cancer [47].

mTOR and cancer

As all cancer types, also hepatocellular carcinoma expresses several kind of growth factors indispensable to sustain uncontrolled cellular proliferation. One of the most largely studied, as one of the most involved in hepatocellular carcinoma genesis and development, is Insulin Growth Factor (IGF), that after binding its specific receptors, localized on cellular

surface, activates intracellular signalling cascade through PI3K-AKT-mTOR (phosphatidylinositol 3-kinase/serine-threonine kinase-mTOR), leading to protein synthesis and influencing a lot of cellular processes including growth, proliferation and nutrient uptake [48]. mTOR is the mammalian target of Rapamycin also known as FK506 binding protein12-rapamycin associated protein 1 (FRAP1) which in humans is encoded by the FRAP gene and is active in 25-45% of hepatocellular carcinoma. mTOR is the catalytic subunit of two molecular complexes: mTORC1 and mTORC2 [49]. mTORC1, defined by the presence of Raptor, is characterized by the classic features of mTOR by functioning as a nutrient/energy/redox sensor and controlling protein synthesis [50-51]. The activity of this complex is stimulated by insulin, growth factors, serum, phosphatidic acid, aminoacids (particularly leucine), and oxidative stress [52]. mTORC1 is inhibited by low nutrient levels, growth factor deprivation, reductive stress, rapamycin, and farnesylthiosalicylic acid (FTS) [53]. The two best-characterized mTORC1 substrates, elongation factor 4e-binding protein 1 (4e-BP1) and ribosomal protein S6 kinase-1 (S6K1), are components of the translational control machinery and mediate cap-dependent translation and ribosome biogenesis, respectively. In addition, mTORC1 negatively regulates autophagy and cellular adaptation to hypoxia. The mTORC2 signalling pathway, instead, is less clearly defined than the mTORC1 signalling pathway, but it has been shown that is an important regulator of the cytoskeleton through its stimulation of F-actin stress fibers, Paxillin, RhoA, Rac1, Cdc42, and protein kinase C α (PKCα) [54]. mTORC2 also appears to possess the activity of a previously elusive protein known as "PDK2". mTORC2 contains three unique proteins: Rictor, mSIN1 and the recently described Protor-1 (also called PRR5). A pivotal upstream signal for mTORC1 activation involves the stimulation of Class I PI3Ks by members of the receptor tyrosine kinase family (Fig.9.1) Activation of PI3K triggers the conversion of phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2) to the bioactive messenger, phosphatidylinositol 3,4,5,-trisphosphate (PtdIns(3,4,5)P3). Elevated levels of PtdIns(3,4,5)P3 drive the plasma membrane recruitment and subsequent activation of

several homology domain-containing proteins, including the protein serine-threonine kinase, AKT. Membrane-localized AKT is subject to two activating phosphorylation events, one (at Thr308) executed by PDK1, and the second (at Ser473) mediated at least in part by mTORC2, as discussed previously [55]. The complexity of the mTOR signalling network is underscored by the observation that mTOR in one complex (mTORC2) is an upstream activator of AKT, whereas, in another guise (mTORC1), mTOR is a downstream recipient of AKT dependent stimulatory signals. Activated AKT stimulates mTORC1 through the heterodimeric Tuberous Sclerosis Complex (TSC), which is a heterodimer that comprises TSC1 and TSC2 subunits [49, 56]. TSC functions as a GTPase activating protein (GAP) for the small, Ras-related GTPase, Rheb (Ras homolog enriched in brain). As a Rheb specific GAP, TSC acts as a signal-modulated suppressor of Rheb by driving Rheb into the inactive, GDP-bound state. According to one model, AKT stimulates the accumulation of GTP-bound Rheb, which in turn promotes the activation of mTORC1 by disrupting the TSC complex through phosphorylation of the TSC2 subunit. Hyperactivation of the PI3K–AKT pathway is a feature of most, if not all types of cancer cells [57]. A frequent cause of deregulated PI3K signaling in human cancer is an acquired deficiency in the phosphoinositide phosphatase, PTEN (phosphatase and tensin homolog deleted on chromosome ten). The lipid phosphatase activity of PTEN reverses the reaction catalyzed by PI3K, converting PtdIns(3,4,5)P3 back to PtdIns(4,5)P2. Loss of PTEN function therefore promotes abnormal PtdIns(3,4,5)P3 accumulation, and in turn, the activation of multiple pro-oncogenic signalling pathways, which include those that involve mTORC1 [58]. Because of mTOR involvement in carcinogenesis and tumoral proliferation [59] its inhibitors have been largely utilized in cancer treatment. Rapamycin, indeed is an immunosuppressant drug used to prevent rejection in organ transplantation. This macrolide, was first discovered as a product of the bacterium *Streptomyces hygroscopicus* in a soil sample from Easter Island (an island also known as Rapa Nui, hence the name) and has been demonstrated to be able to inhibit mTORC1. HCC is a complex disease with

multiple underlying pathogenic mechanisms caused by a variety of risk factors, additionally, as discussed here, the expression of a large number of genes, proteins, and other molecules belonging to diverse cellular processes and pathways are altered in HCC. In this study we want to evaluate the somatostatin and mTOR role in molecular mechanisms at basis of hepatocarcinogenesis and their potential therapeutic effects.

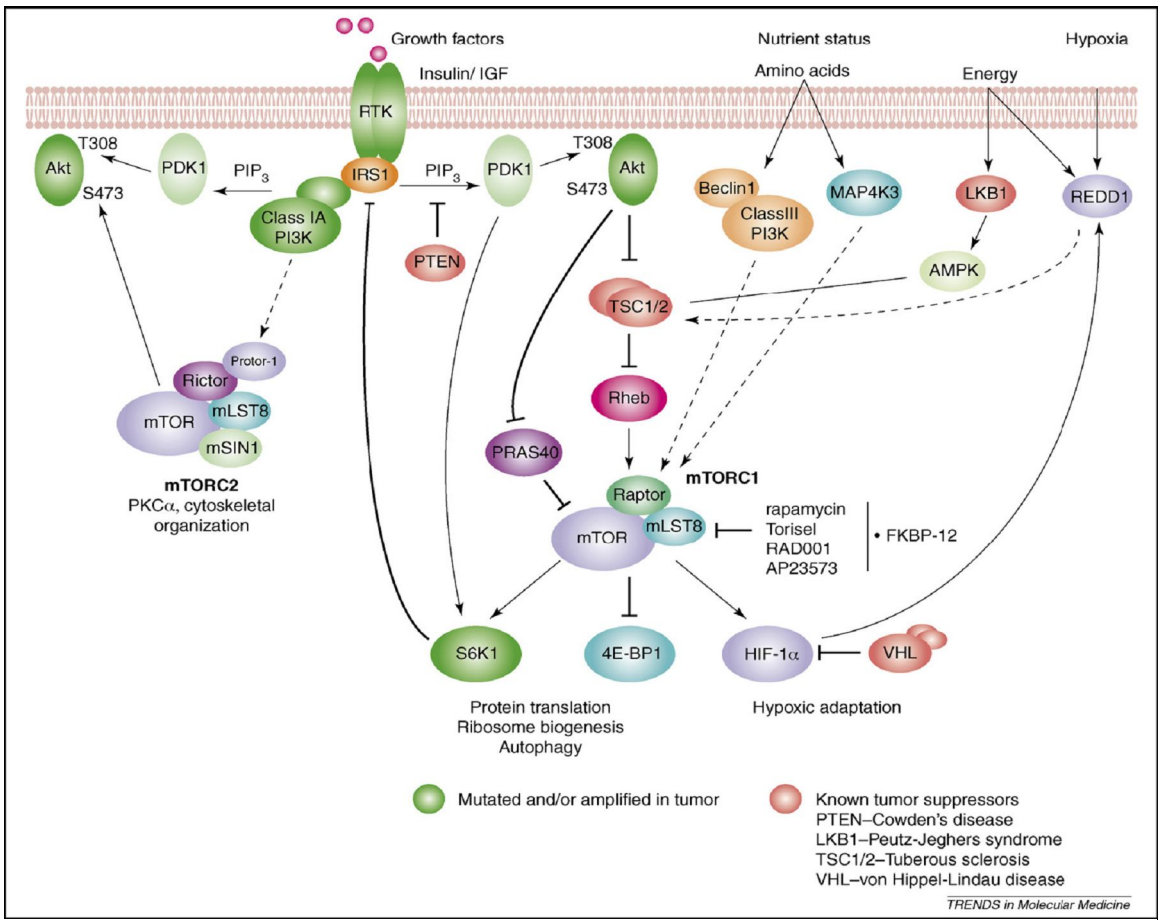


Fig.9.1: The mTORC1 complex (mTOR, mLST8 and Raptor) signals to 4E-BP1, p70S6K and HIF-1, which mediate cap-dependent translation, ribosome biogenesis, and hypoxic adaptation, respectively**.** The rapalogs (Torisel, RAD001, and AP23573) bind to the immunophilin, FKBP12, to generate highly potent and specific inhibitors of mTORC1 dependent signalling. The mTORC2 complex (mTOR, mLST8, Rictor, mSIN1 and Protor-1) is insensitive to the Rapalogs, and regulates AKT Ser473 phosphorylation, PKC-a phosphorylation, and cytoskeletal organization. In figure, arrows represent activation whereas bars represent inhibition. Broken lines indicate pathways that are poorly defined.

Aim of the thesis

The growing incidence of HCC has generated intense research to understand the physiological, cellular and molecular mechanisms of this disease with the hope of developing new treatment strategies. Our molecular study focused on the identification of a potential therapeutic role of somatostatin analogues, dopamine agonists and mTORC1 inhibitors in HCC and CC. We based our rationale on the assumption that neuroendocrine tumors represent a peculiar type of tumors characterized by the production of growth factors and the expression of somatostatin receptors [60] [61], so if HCC and CC have neuroendocrine characteristics the aims of the current study are to evaluate the expression level of the five somatostatin receptors by real-time RT-PCR in a series of HCC, in two hepatocellular cell lines (HepG2 and HuH-7), and in two cholangiocarcinoma cell lines (TFK-1 and EGI-1) and to evaluate *in vitro* effects of somatostatin analogues, dopamine agonists and mTORC1 inhibitors on functional assays. For this purpose we evaluated, as first step of our investigation, the receptorial profile with Real-Time PCR on 23 Caucasian patients with untreatable HCC and simultaneously, the same study has been led on two different HCC and CC cell lines. In HepG2 and HuH-7 we confirmed this molecular expression through immunocytochemistry analysis also. In the second step of our study, we focused our attention expecially on sstr2 and sstr5 being the receptors mainly involved in antiproliferative effects, but also on sstr1. At first we assessed the sst2-specific and non-specific agonist ability to induce receptor internalization due to the phosphorylation by G-protein coupled receptor kinases (GRKs) followed by recruitment of ß-arrestin which internalizes together with the receptor into the cytoplasm in HuH-7. Then, we evaluated the role of all receptor ligands, as potential therapeutical compounds, such as Octreotide, Pasireotide, Bim 23244, Bim 23926, Bim 23206, in cellular proliferation and secretion. To test the effect of ligand post-stimulus reaction, AFP (the actually accepted tumor biomarker for HCC) and cell proliferation were tested on all four cell lines. Of all secreted markers (CEA, CA19-9 and IGF1) evaluated in CC cell lines, none was produced by the cells

themselves. Moreover, this molecular study has also been extended to mTOR inhibitors evaluating, on the same cell lines, the antiproliferative effects of Rapamicyn, Temsirolimus and Everolimus in a dose-time dependent manner, with the purpose to find the optimal conditions of treatment. Furthermore, we investigated the intracellular molecular phosphorilation change of ERK1/ERK2 involved in proliferation mechanisms by Western Blot analysis also to define the relationship between mTOR pathway and somatostatin signalling pathway.

Results

Receptor expression in hepatocellular carcinoma samples and cell lines

23 caucasian patients with untreatable HCC were enrolled in the study. The characteristics of patients are shown in Table 3.1. The median age was 73 years and the majority of patients were male. 14 patients (61%) are classified as degree 3 of Child-Pugh classification, showing a decompensated disease, 2 patients (8.7%) show a well-compensated disease classified as degree 1, and 4 patients (17.4%) had a score belonging to degree 2 with functional activity significantly compromised. HCV or HBV hepatitis are the main cause of cirrhoris that leads to the development of HCC, the 78.3% of patients displayed HCC related to HCV infection and the 8.7% HCC related to HBV infection. The percentage of gene expression in our population is summarized in Fig. 3.1. The 100% of patients expressed somatostatin receptor type 2. Sstr1 was expressed in the 91.3% of cases; sstr5 in the 26.1% and D2R in the 21.7% . The mRNA relative expression levels of SSTRs, and D2R in hepatocellular carcinomas are shown as mean in Fig. 3.2. Among the investigated somatostatin receptor subtypes sstr2 was the most predominantly expressed with a mean of 0.0855±0.032 (median: 0.032). Sstr1 was expressed at lower levels with a mean of 0.0331±0.006 (median: 0.023); also sstr5 was expressed at low levels with a mean of 0.00634±0.0045 (median: 0). Sstr3 was no detectable in this group of patients. About the dopamine receptor, D2R was present but its levels were very low with a mean of 0.0009±0.0003 (median: 0.0005). The univariate correlation analysis between the expression levels of the individual genes revealed no positive correlation between them. Moreover, we performed the correlation, using a linear regression, between the expression levels of the individual somatostatin and dopamine receptors and the principal clinical (Child-Pugh classification), biochemical (AFP values) and aetiopathogenetic parameters (Hepatitis infections). None of these variables were significantly associated with the expression of any of the investigated receptor subtypes (data not shown).

The two hepatocellular cell lines showed the same receptor profile but with a different amount in the expression levels of somatostatin receptors. HepG2 and HuH-7 were positive for the sstr1, sstr2, sstr5 and negative for D2r. HepG2 displayed high levels of sstr5 and low levels of sstr1 and sstr2, instead HuH-7 showed high levels of sstr1 and sstr2 with a low expression level of sstr5 (Fig. 3.3). TFK-1 cell line was positive for sstr1 at high levels, and sstr2, instead EGI-1 cell line showed only very low levels of sstr2 (Fig. 3.4). For the analysis of mTOR pathway we evaluated the mRNA expression levels of mTOR and its downstream activated proteins such as 4e-BP1 and p70S6K. As shown in Fig. 3.5, HepG2, HuH-7, TFK-1 and EGI-1 cell lines express the same molecular profile, with high levels of 4e-BP1 copies mRNA/HPRT and low levels of mTOR and p70S6K.

Characteristics	**n**	**%**
Sex		
Male	18	78.3
Female	5	21.7
Child-Pugh class		
A	2	8.6
B	4	17.4
C	17	74
Noduls		
1	1	4.4
2	22	95.6
Portal thrombosis		
yes	10	43.5
no	13	56.5
Virus infections		
HCV^+	21	91.3
HBV^+	2	8.7

Table 3.1: baseline characteristics of HCC patients. HCV= hepatitis C virus; HBV= hepatitis B virus.

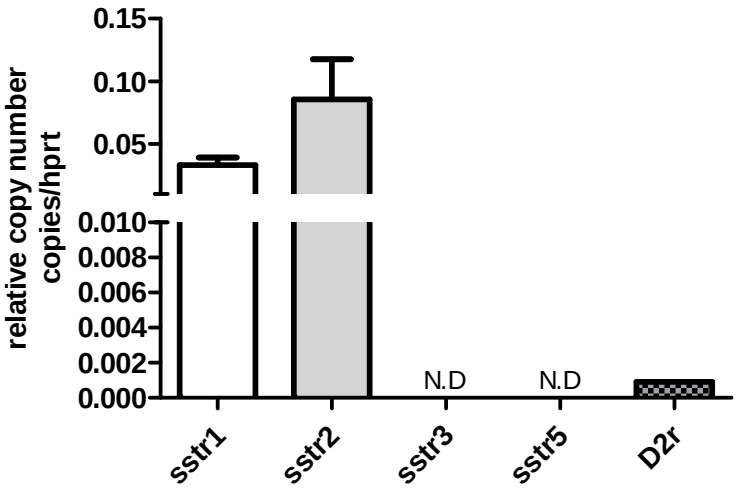


Fig: 3.1: Mean expression levels of somatostatin receptor subtypes and D2 receptor in 23 human hepatocellular carcinomas. Values represent the mean ± SEM per gene, assayed in duplicate. Expression levels are normalized against the housekeeping gene HPRT. N.D.: "Not detectable."

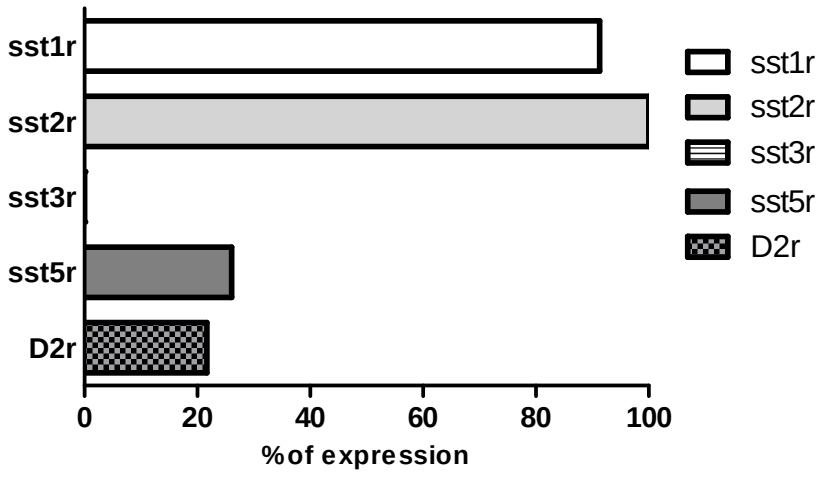


Fig: 3.2: Percentage of gene expression in the population of 23 human hepatocellular carcinoma.

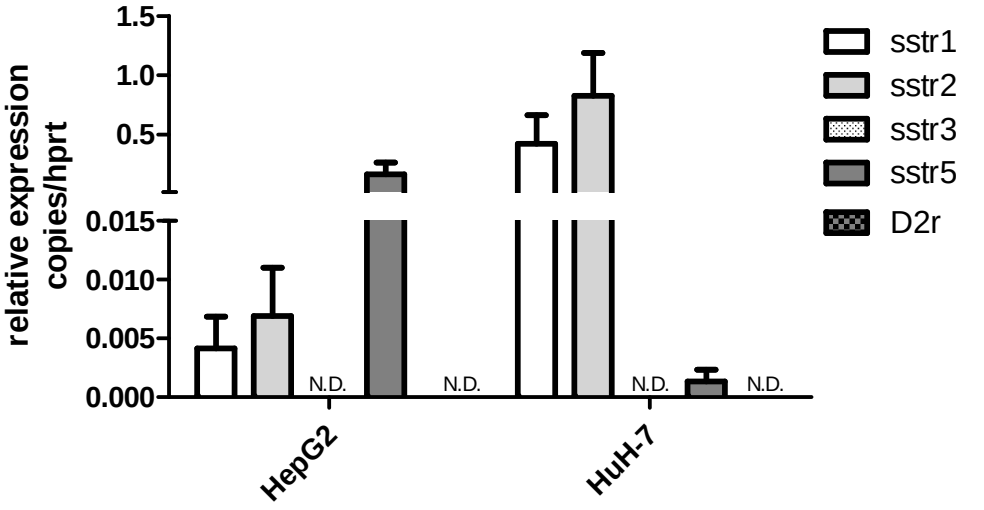


Fig: 3.3: Mean expression levels of somatostatin subtype receptors in human hepatocellular cell lines. Values represent the mean ± SEM per gene assayed in duplicate in two independent experiments. Expression levels are normalized against the housekeeping gene HPRT. N.D. "Not detectable"

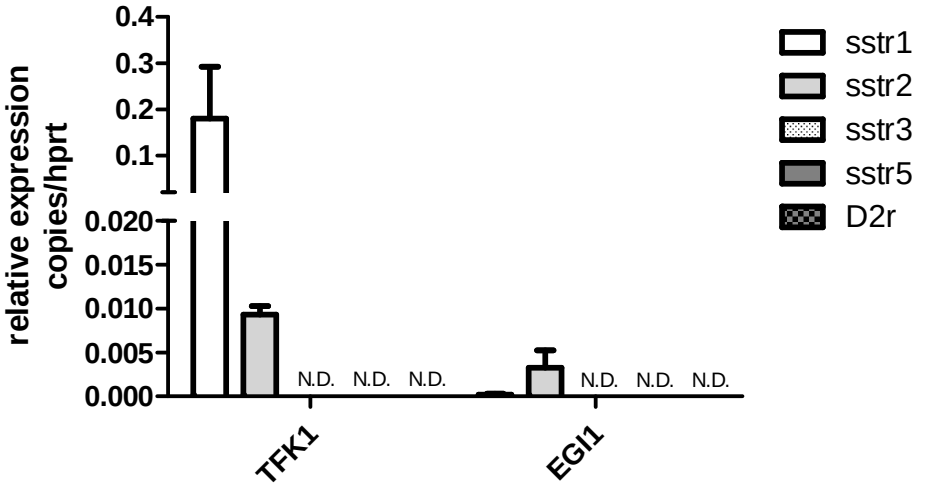


Fig: 3.4: Mean expression levels of somatostatin subtype receptors in human cholangiocellular cell lines. Values represent the mean ± SEM per gene assayed in duplicate in two independent experiments. Expression levels are normalized against the housekeeping gene HPRT. N.D. "Not detectable"

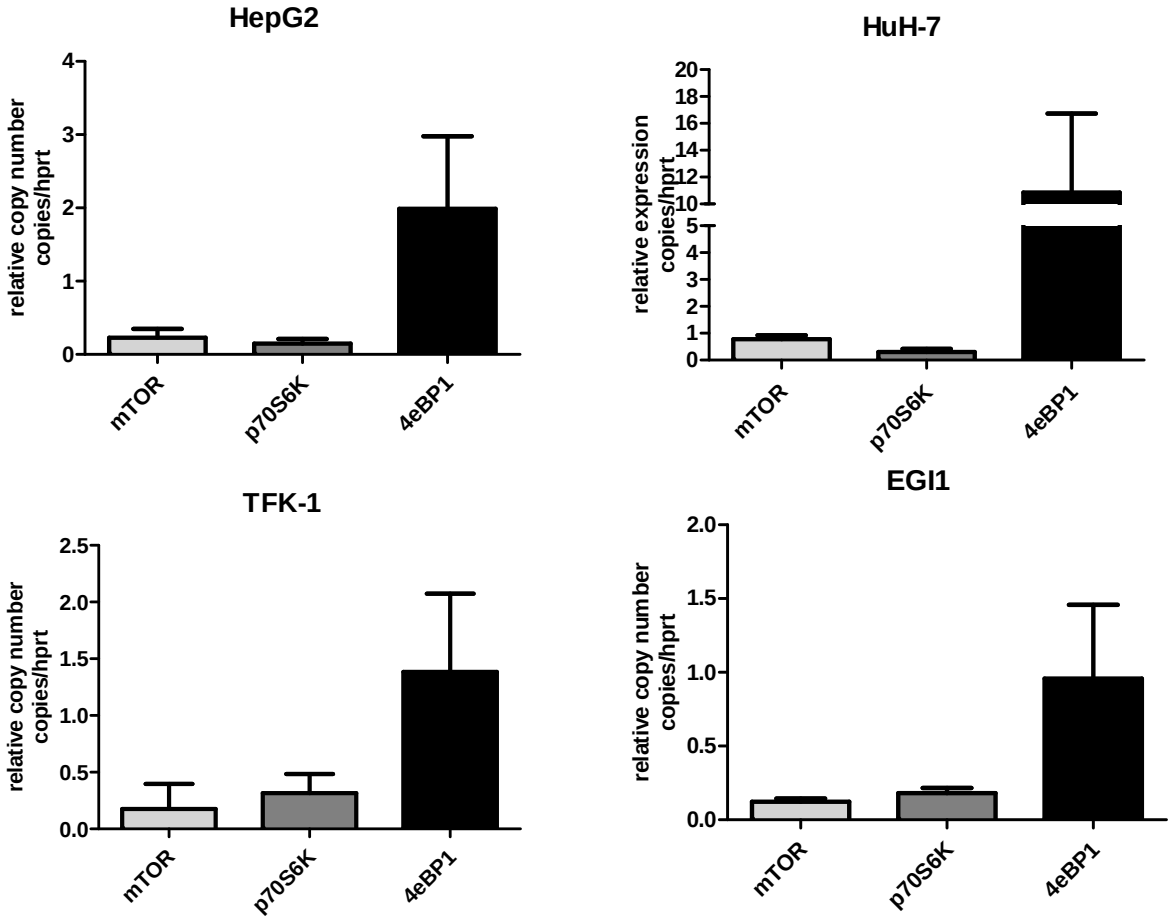


Fig. 3.5: mean expression levels of mTOR, p70S6K and 4e-BP1 molecules in HepG2, HuH-7, TFK-1 and EGI-1 cell lines. Values represent the mean ± SEM per gene, assayed in duplicate. Expression levels are normalized against housekeeping gene HPRT.

Immunocytochemical and western blot analysis

We also investigated sstr2 and sstr5 expression in HepG2 and HuH-7 cell lines with an experiment of immunocytochemistry. In basal condition, both cell lines showed an heterogeneous staining, with a subpopulation of cells positive to the investigated receptor subtypes (Fig. 3.6). HepG2 cell line expressed sst5 receptor, localized at cytoplasmatic level, as shown in the detail of Fig. 3.6E and F, but not sst2 receptor (Fig. 3.6A and B). In HuH-7, membrane and cytoplasmic staining was seen for sst2 receptor (Fig. 3.6C and D) while the majority of the cells positive to the staining mainly showed a membrane localization for the sstr5 subtype but also located in intracellular components, as indicated

by the arrows (Fig. 3.6G and H). Moreover, the treatment with SS-14 and somatostatin analogs at 10^{-6}M induced internalization of sst2 receptor in HuH-7, showing a post-stimulus reaction (Fig. 3.7 A-F).

Negatively surprised by the absence of sstr2 staining in HepG2, we decided to investigate better the presence of sstr2 protein in HepG2 and HuH-7 cell lines through a Western Blot Analysis using a specific antibody capable to recognize this receptor subtype. As reported in the Fig. 3.8, both cell lines expressed the receptor. TT and H727 cell lines were used as controls. Different molecular weights in kD are due to the different glycosylation degree of the receptors.

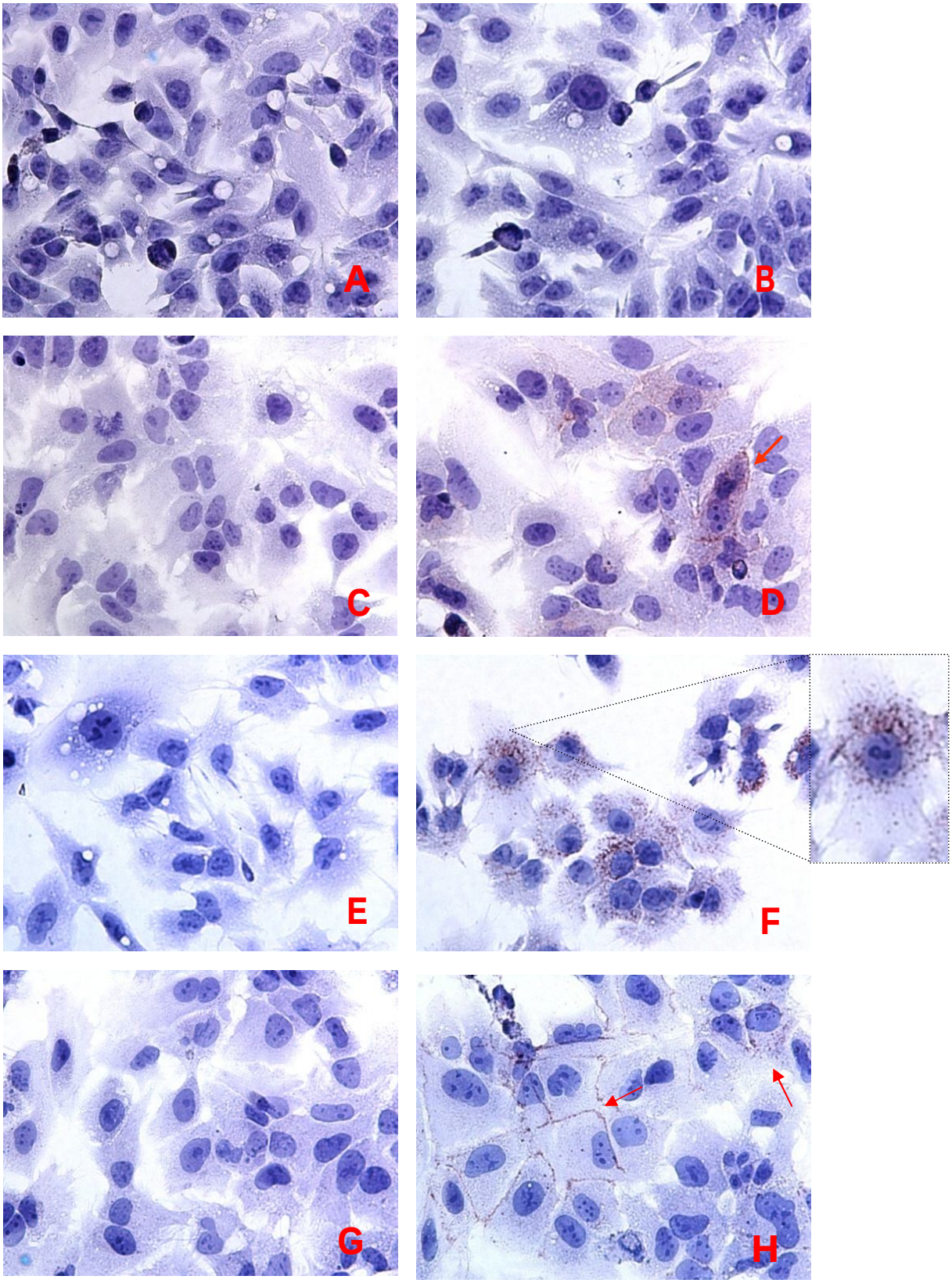


Fig. 3.6: Immunocytochemestry in basal condition in HepG2 and HuH-7 cell lines. A) Negative Control for sstr2 in HepG2. B) sstr2 in HepG2. C) Negative control for sstr2 in HuH-7. D) sstr2 in HuH-7. E) Negative control for sstr5 in HepG2. F) sstr5 in HepG2. G) Negative control for sstr5 in HuH-7. H) sstr5 in HuH-7.

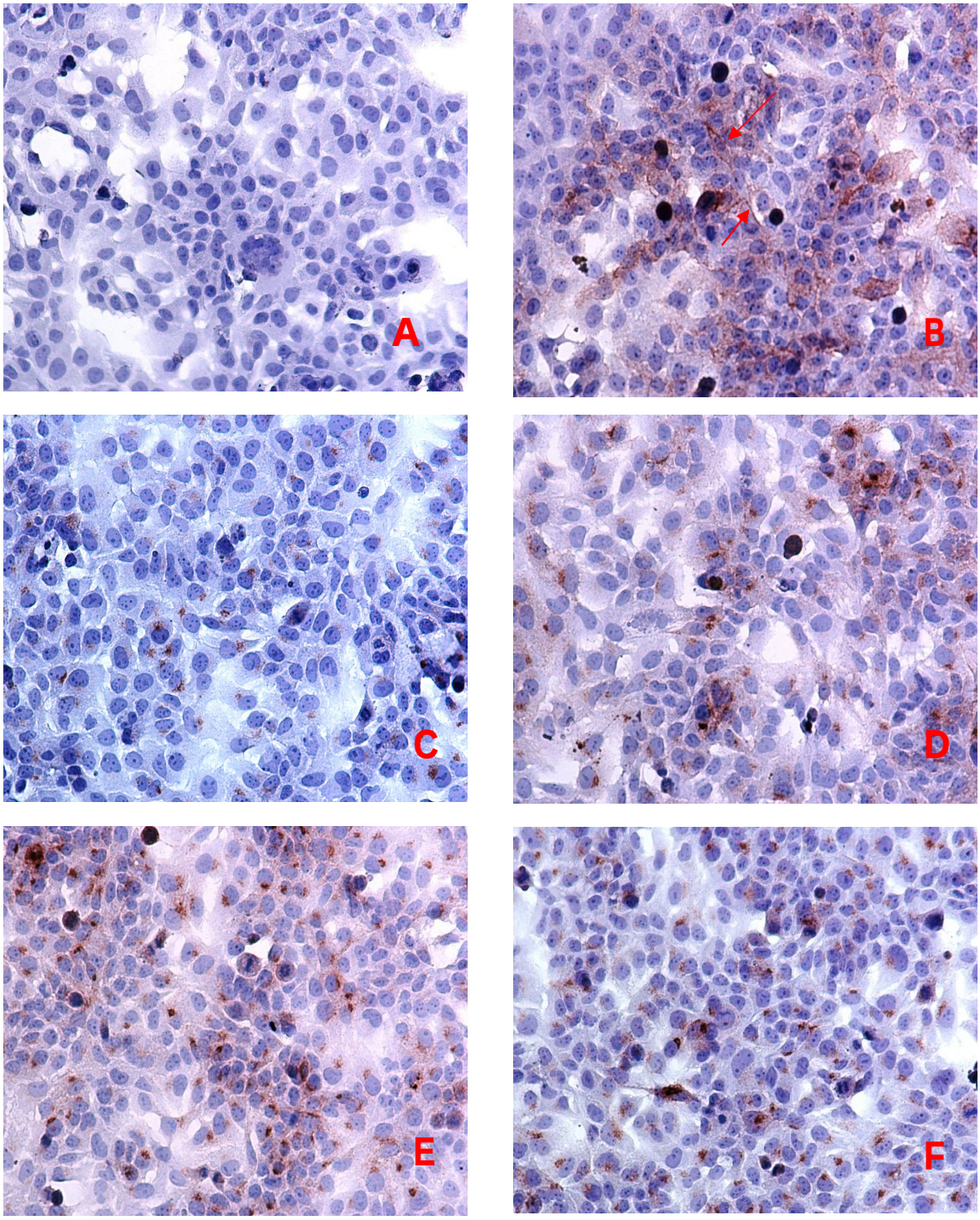


Fig. 3.7: Immunocytochemestry for sstr2 in basal condition and after treatment of 30 min with several somatostatin analogs in HuH-7 cell lines. A) Negative Control for sstr2. B) sstr2 without treatment. C) sstr2 in presence of Octreotide 10^{-6}M. D) sstr2 in presence of Bim 23779 10^{-6}M. E) sstr2 in presence of pasireotide (Som 230) 10^{-6}M. F) sstr2 in presence of SS14 10^{-6}M.

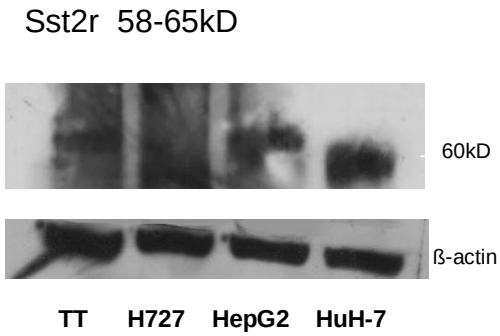


Fig. 3.8: Sstr2 protein expression in HepG2 and HuH-7. TT and H727 are two different neuroendocrine cell lines used as control. The protein expression levels were normalized against ß-actin.

Cell proliferation assay

All four cell lines were subjected to escalating doses of somatostatin analogues to assess whether this compounds exert an antiproliferative effect on hepatocarcinoma and on cholangiocarcinoma. Given the somatostatin receptor profile of HCC and CC cell lines, we tested different somatostatin analogs: octreotide, SOM230, Bim 23926, Bim 23244 and Bim 23206 for HCC cell line and octreotide, Bim 23926 and Bim 23779 for CC cell lines. As shown in Fig. 3.9, Octreotide does not produce a significant antiproliferative effects in both cell lines, showing a slight no significant 15% of inhibition in HuH-7 and 10.3% in HepG2 at concentration of 10^{-7}M. Bim 23244 showed a significant inhibition (18.2%) at 10^{-7}M concentration only in HepG2 cell line; moreover, also Bim 23926 exercised a proliferation inhibition (16.4%) in HuH-7 at concentration of 10^{-7}M. The universal ligand, Pasireotide and Bim 23206 had no effect on both cell lines. As shown in Fig. 3.10, in EGI-1 cell line we just tested Bim 23779, another universal ligand, with no effect; also in TFK-1 Bim 23926, Bim 23779 and octreotide had no effect on proliferation inhibition.

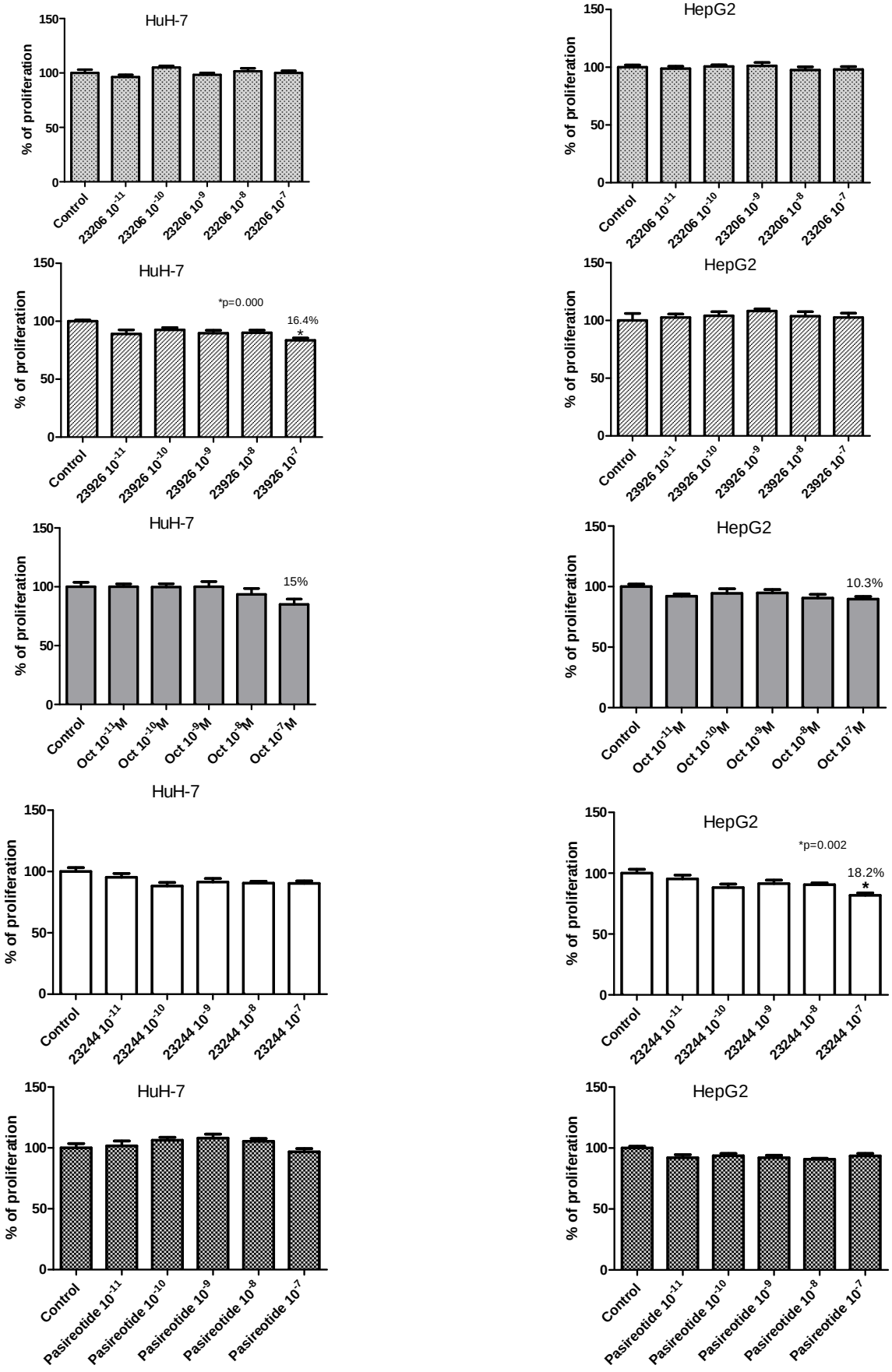


Fig. 3.9: Proliferation assay in HuH-7 and HepG2 cell lines. The cells were subjected to escalating doses of drugs for 72h in medium with 1% of FBS. In each experiment, the treatment was in quadruplicates and the graphs represent the mean of three independent experiments.

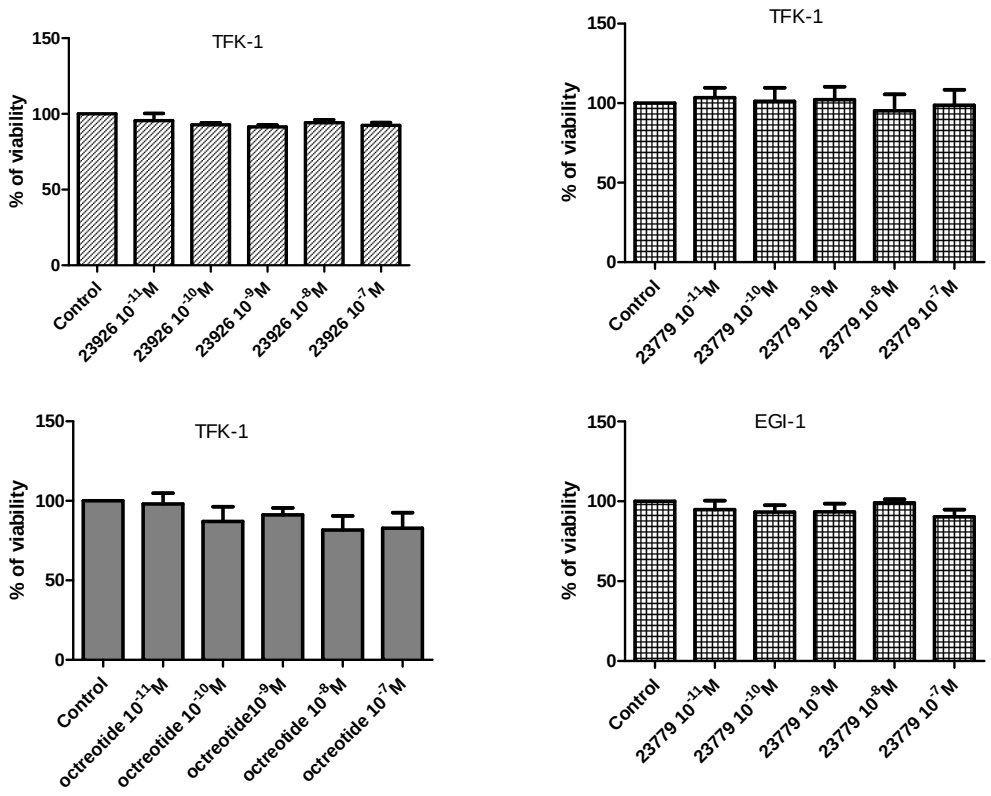
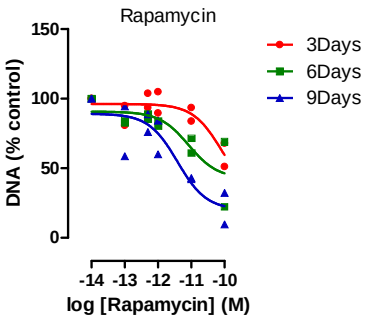


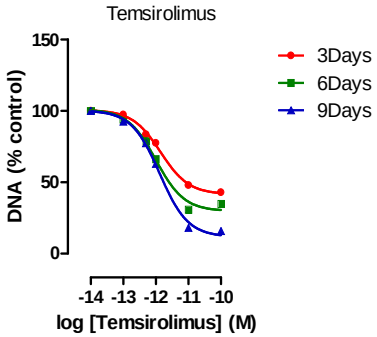
Fig. 3.10: Proliferation assay in TFK-1 and EGI-1 cell lines. The cells were subjected to escalating doses of drugs for 72h in medium with 1% of FBS. In each experiment, the treatment was in quadruplicates and the graphs represent the mean of three independent experiments.

Furthermore, all investigated cell lines were subjected to escalating dose of mTOR inhibitors: Rapamycin, Everolimus and Temsirolimus and on the same cells a DNA assay were employed to evaluate cellular proliferation after 3, 6 and 9 days of contact with the drugs. As shown in Fig. 3.11 A-B-C (HepG2), 3.11 D-E-F (HuH-7), 3.11 G-H-I (TFK-1) and 3.11 L-M-N (EGI-1), all mTOR inhibitors induce a dose-time dependent inhibition of proliferation in HCC cell line and TFK-1 cell line, while EGI-1 cell line seems to be resistant to the treatment especially after 9 days, that's why for this cell line it was impossible to calculate the drug IC50.



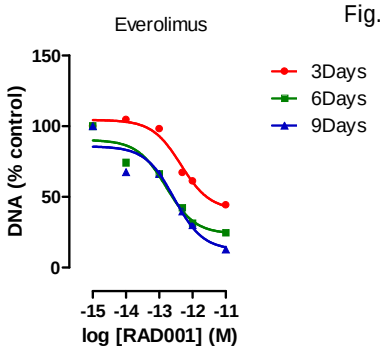
	3Days	6Days	9Days
IC50	8.349e-011	8.324e-012	3.860e-012

Fig. 3.11 A



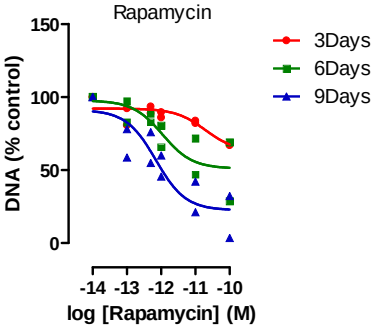
	3Days	6Days	9Days
IC50	1.402e-012	1.014e-012	1.318e-012

Fig. 3.11 B



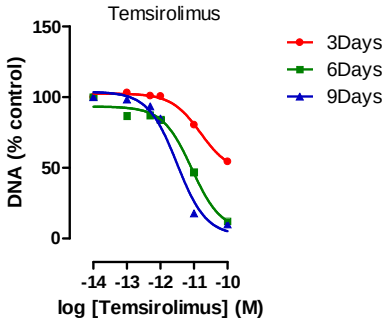
	3Days	6Days	9Days
IC50	4.512e-013	1.599e-013	2.789e-013

Fig. 3.11 C



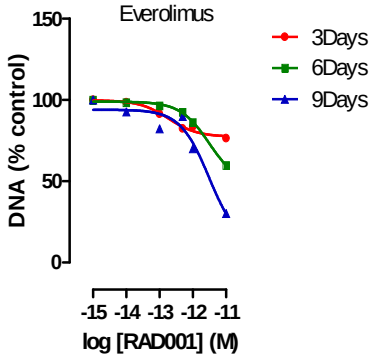
	3Days	6Days	9Days
IC50	1.947e-011	1.098e-012	7.034e-013

Fig. 3.11 D



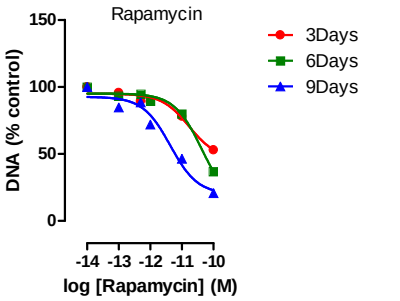
	3Days	6Days	9Days
IC50	1.571e-011	8.658e-012	3.139e-012

Fig. 3.11 E



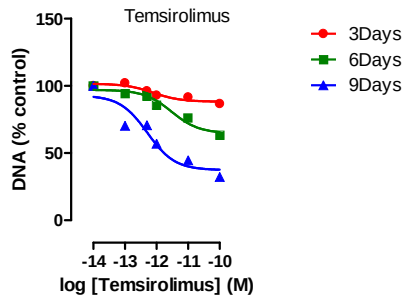
	3Days	6Days	9Days
IC50	1.865e-013	3.119e-012	3.098e-012

Fig. 3.11 F



	3Days	6Days	9Days
IC50	1.694e-011	4.239e-011	4.304e-012

Fig. 3.11 G



	3Days	6Days	9Days
IC50	7.834e-013	2.979e-012	5.134e-013

Fig. 3.11 H

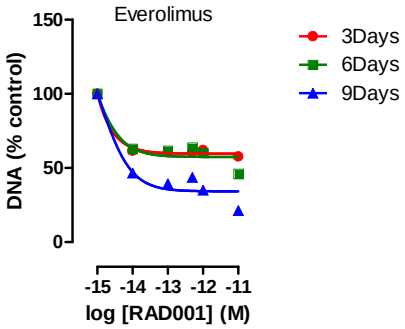


Fig. 3.11 I

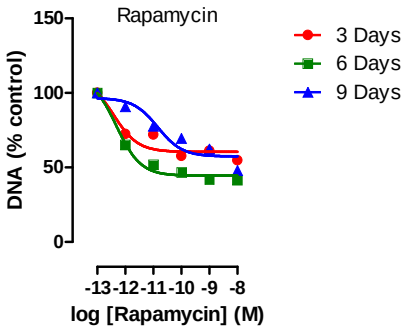


Fig. 3.11 L

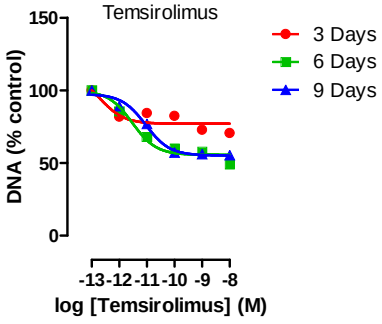


Fig. 3.11 M

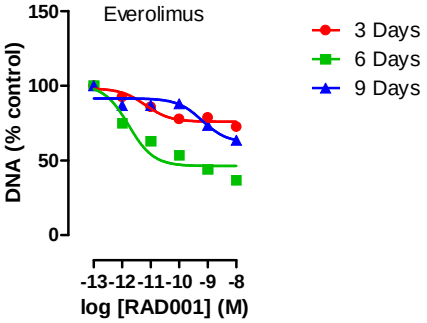


Fig. 3.11 N

Fig. 3.11: Proliferation assay in HepG2 (A-B-C), HuH-7 (D-E-F), TFK-1 (G-H-I) and EGI-1 (L-M-N) cell lines. The cells were subjected to escalating doses of mTOR for 3, 6 and 9 days. In each experiment, the treatment was performed in quadruplicates and the graphs represent the mean of three independent experiments.

In order to investigate the effect of combined treatment of somatostatin analogs and mTOR inhibitors, we performed a proliferation assay in HepG2 and HuH-7 cell line in which the cells were subjected to high dose of octreotide plus rapamycin at different doses (10^{-8} and 10^{-12} M). Surprisingly as shown in Fig. 3.12, in both cell line when rapamycin was inoculated at low dose, octreotide was able to revert the inhibitory effect induced by rapamycin suggesting a molecular escape set out by an intelligent tumoral cell. This effect is also correlated to an up-regulation of pMAPK, proteins involved in survival pathways.

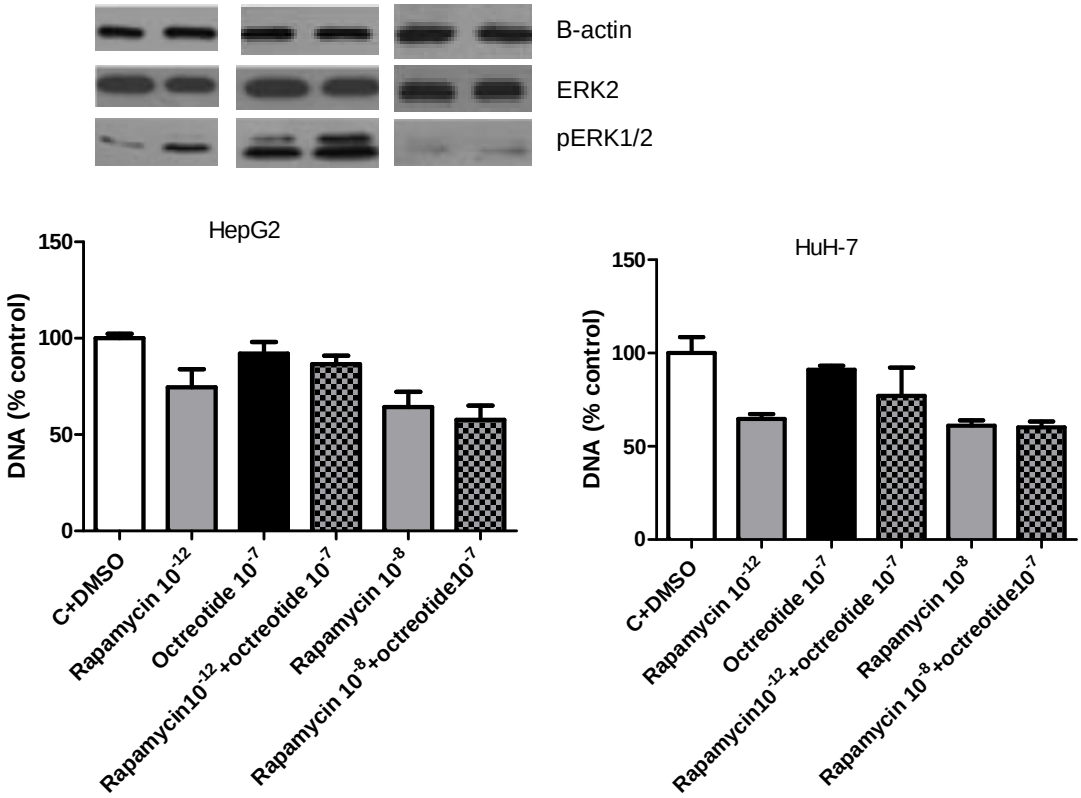


Fig. 3.12: Proliferation assay in HepG2 and in HuH-7 cell lines. The cells were subjected to rapamycin and octreotide for 3 days. In each experiment, the treatment was performed in quadruplicates and the graphs represent the mean of three independent experiments.

Given these results, we evaluated the somatostatin receptor expression in HepG2 cells conditioned by rapamycin. The treatment of 30' with rapamycin 10^{-10}M leads to an increase of sstr2 and sstr5 mRNA levels, but after 2 hours, this effect tends to decrease (Fig. 3.13).

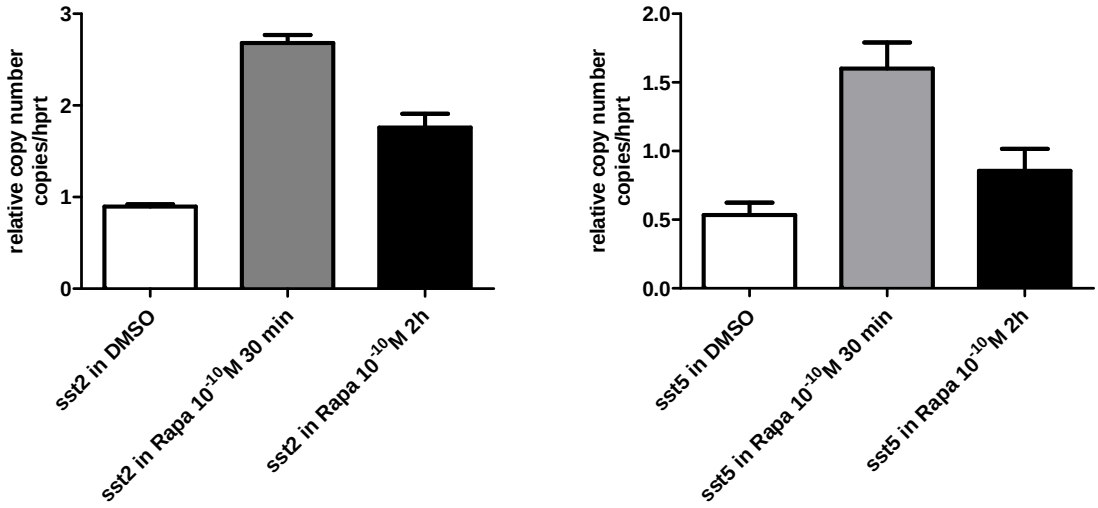


Fig. 3.12: Receptor expression in HepG2 after treatment with rapamycin.

Propidium iodide staining

To determine whether somatostatin analogs induce apoptosis, propidium iodide staining was assessed only on HepG2 cells. HepG2 were exposed at somatostatin analogs at concentrations of 10^{-7}M for 72h and after incubation HepG2 have been harvested and examined for DNA content. Apoptosis was investigated by DNA distribution as revealed by flow cytometry demonstrating hypodiploid DNA and as shown in Fig. 3.13, when all used somatostatin analogs had no effect on cell apoptosis on HepG2 compared with unstimulated cells, confirming the cell proliferation assay.

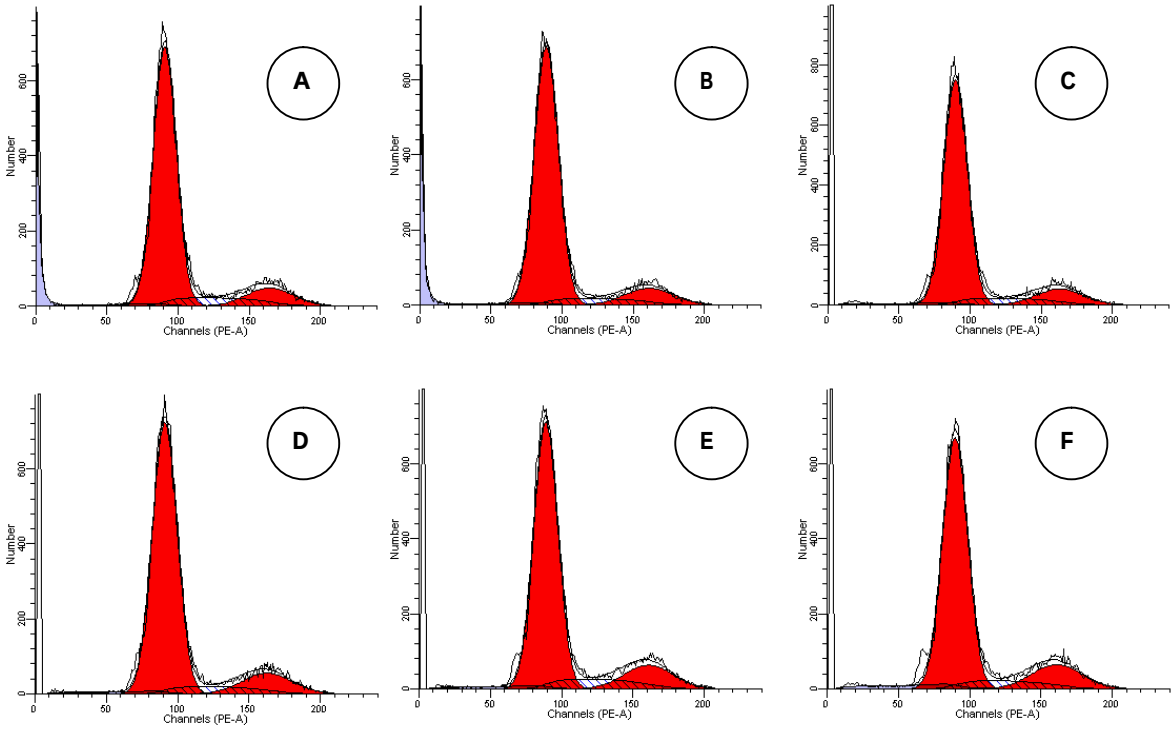
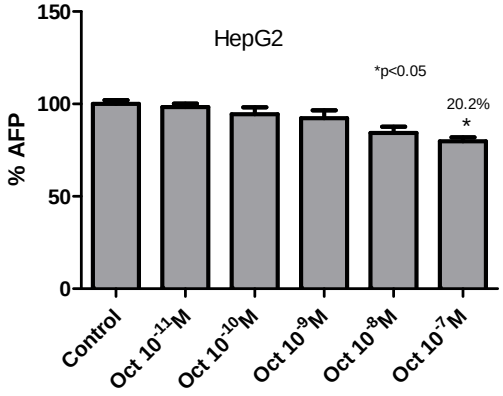
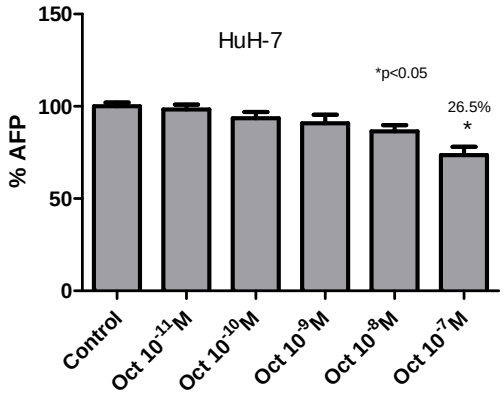


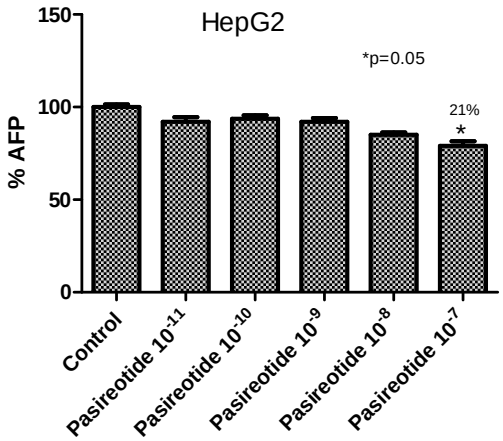
Fig. 3.13: Flow citometry on serum free HepG2 cell line: A) Control, B) Octreotide 10^{-7}M, C) Bim 23244 10^{-7}M, D) Bim 23206 10^{-7}M, E) Bim 23926 10^{-7}M, F) Pasireotide 10^{-7}M.

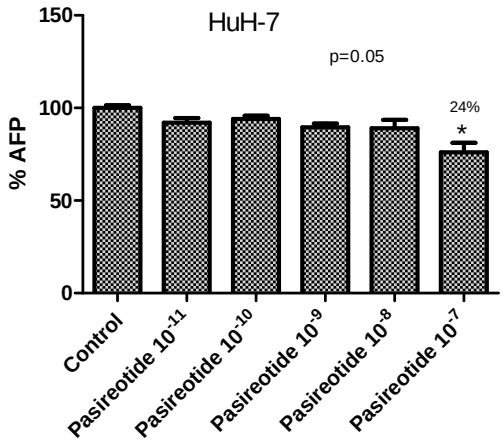
AFP assay

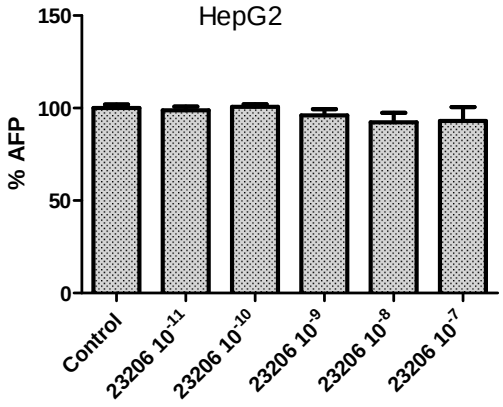
To evaluate somatostatin analogues antisecretive effects, the supernatant of the same cell cultures used in the proliferation assay, were subjected to AFP measure using an immuno-chemiluminescent assay (CLIA) (Fig. 3.14). In HepG2, octreotide, pasireotide and Bim 23244 induced a slight inhibition of AFP secretion. In HuH-7, octreotide, pasireotide caused a slight significant inhibition while Bim 23926 produced an no significant inhibition of 17% at high concentration.

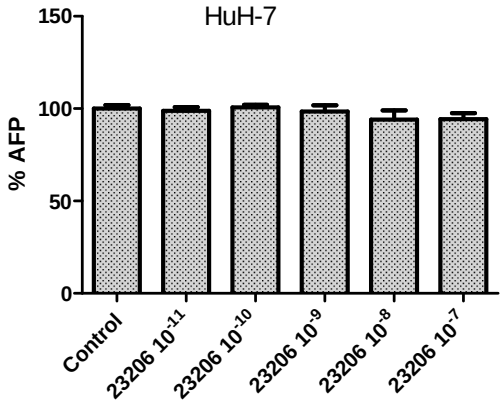

HepG2
*p<0.05
20.2%
*
% AFP
150
100
50
0
Control
Oct 10-11M
Oct 10-10M
Oct 10-9M
Oct 10-8M
Oct 10-7M

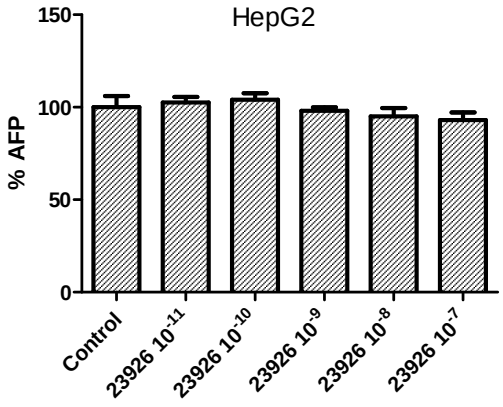

HuH-7
*p<0.05
26.5%
*
% AFP
150
100
50
0
Control
Oct 10-11M
Oct 10-10M
Oct 10-9M
Oct 10-8M
Oct 10-7M

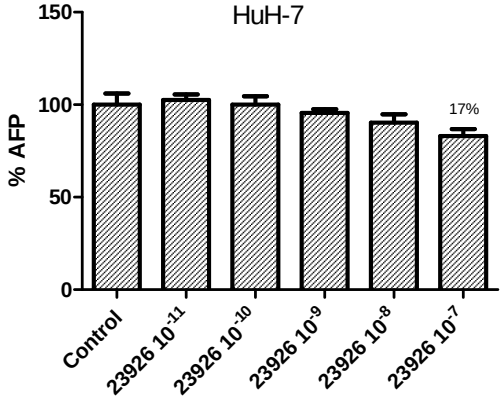

HepG2
*p=0.05
21%
*
% AFP
150
100
50
0
Control
Pasireotide 10-11
Pasireotide 10-10
Pasireotide 10-9
Pasireotide 10-8
Pasireotide 10-7


HuH-7
p=0.05
24%
*
% AFP
150
100
50
0
Control
Pasireotide 10-11
Pasireotide 10-10
Pasireotide 10-9
Pasireotide 10-8
Pasireotide 10-7


HepG2
% AFP
150
100
50
0
Control
23206 10-11
23206 10-10
23206 10-9
23206 10-8
23206 10-7


HuH-7
% AFP
150
100
50
0
Control
23206 10-11
23206 10-10
23206 10-9
23206 10-8
23206 10-7


HepG2
% AFP
150
100
50
0
Control
23926 10-11
23926 10-10
23926 10-9
23926 10-8
23926 10-7


HuH-7
17%
% AFP
150
100
50
0
Control
23926 10-11
23926 10-10
23926 10-9
23926 10-8
23926 10-7

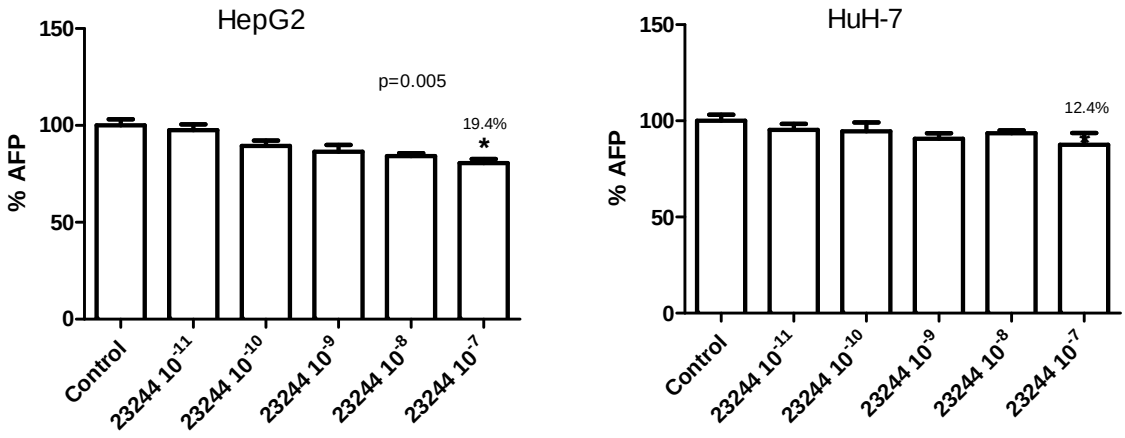


Fig. 3.14: AFP assay on serum free HepG2 and HuH-7 cell line stimulated with different somatostatin analogs at 10^{-7}M concentration.

Discussion and conclusions

HCC and CC are asymptomatic cancers often diagnosed at advanced stages or even in metastatic condition, so liver transplantation or resection cannot be considered as a therapeutic options. Other therapies, such as transcatheter arterial chemoembolization (TACE), radiofrequency ablation (RFA) or photodynamic therapy (PDT) are usually limited to a subgroups of people with small tumors or with a well-preserved liver function tumors [66]. Moreover, therapy with cytotoxic drugs is poorly tolerated by cirrhotic patients. Actually, the molecular targeted therapy has emerged as one of the most important therapy of cancer treatment and currently between the available therapies for the treatment of HCC, Sorafenib (multikinase inhibitor) is the only one approved by the Food and Drug Administration (FDA) [67]. In the field of new therapeutic strategies, the introduction of somatostatin analogs represents an attractive perspective because of the somatostatin ability to inhibit the secretion and growth in a number of neuroendocrine tumors [68], in addition, the peptide receptor target therapy, based on the fact that some receptors are overexpressed in several tumor types and can be targeted by their ligands, has been already used in the past for the development of peptide as carriers of radionuclides in radiotherapy. Just because the role of somatostatin receptors in HCC is not well understood, as demonstrated by conflicting results of several in vivo studies [36-39], and since dopamine receptors have never been investigated in this kind of neoplasia, all these observations provided the rationale for our study. We demonstrated the expression of somatostatin receptors in our HCC group at mRNA level, with a relative expression of sstr2 and sstr1 higher than sstr5. These findings are partially agree with the results of study of Reynaert et al. [42] in which they also found sstr3 in their samples, even though we performed qRT-PCR using different primers already validated and employed in previous studies [69-70]. However, the somatostatin mRNA detection did not correlate with clinical, biochemical and aetiopathogenetic parameters, corroborating the previous

immunohistochemical data [24]. The hepatocellular carcinoma cell lines, HepG2 and HuH-7 showed a super imposable expression profile with presence of sst1, sst2 and sst5 receptor subtypes but, contrary to our expectations, Japanese cell line displayed an expression profile similar to the our investigated Caucasian group of patients. TFK-1 and EGI-1, cholangiocarcinoma cell lines, showed the expression of sstr1 and 2 receptors at very low levels, expecially for EGI-1 cell line. As a result of the characteristics of G-protein coupled receptor (GPCR) heterodimerization [46, 71], new chimeric molecules, binding in specific manner different somatostatin receptors, have been synthesized. The use of chimeric compound with high affinity for sstr2, sstr5 and D2R has been proven beneficial to regulate the production of hormone in acromegaly [72] but also to inhibit the cell proliferation in lung cancer [73], so we focused our attention on these three receptors, especially on sstr2 subtype that is the most frequently expressed in several tumors. Unfortunately, D2R seems to be not expressed in investigated cell lines, so they are not a good models for studying dopaminergic system. Instead, immunocytochemestry results confirmed the presence of sstr5 but not of sstr2 in HepG2 in spite of HuH-7 cell line expressing both sstr2 and sstr5 at protein level, moreover the staining stressed an heterogeneous receptor distribution with cellular subpopulation strongly positive for the staining compare to cells completely negative. These results raise the question about the ability of some cells to acquire a neuroendocrine phenotype but also the possibility that these receptors represent a marker of stem cell membrane as already demonstrate for hematopoietic progenitor cells [74]. The 21.7% of patients showed the presence of D2R but, unfortunately, all four cell lines did not express D2R, showing that they are not a good in vitro models for studying the dopaminergic system in HCC and CC. The use of somatostatin analogs triggers the internalization of sstr2 due to the phosphorylation by G-protein coupled receptor kinases (GRKs) followed by recruitment of ß-arrestin which internalizes together with the receptor into the cytoplasm [33, 75] as also shown by our immunocytochemical data in HuH-7. To test the effect of this post-stimulus reaction, AFP,

the actually accepted tumor biomarker for HCC, and the cell proliferation were tested. No growth factors or tumor markers have been investigated in cholangiocarcinoma cell line because they do not secrete IGF1 or typical tumor markers as CEA or CA19-9. Bim 23206 and Pasireotide didn't show any effect on cell proliferation in HCC cell lines. Octreotide in HuH-7 and HepG2, as well as Bim 23244 in HuH-7 showed a limited and non significant antiproliferative effects. Whereas Bim 23244 induced an 18.2% of inhibition in HepG2, and we can speculate that this drug could trigger a different signalling compare to the mono-specific compound for the sstr5. Also Bim 23926 exercised a limited inhibition (16.4%) only in HuH-7 in which sstr1 mRNA expression was higher than in HepG2 cell line; this result open a new scenario on the role of sst1 receptor in the regulation of proliferation as already demonstrated in other tumor types [76-77]. Also flow citometry analysis confirmed on HepG2 that somatostatin analogs had no effects on cell proliferation and apoptosis. These data are not in agreement with the findings of Tsagarakis [78], who demonstrated with flow cytometry a significant apoptosis induced by octreotide on HepG2 cells. Octreotide, Bim 23926 and Bim 23779 had no effect on cholangiocellular cell lines. In publication no one work is actually present about the activity of mTOR inhibitors on HCC and CC cell line proliferation. Incubation of FBS-cultured cells with mTOR inhibitors for 3, 6 and 9 days resulted in a dose-dependent growth inhibition in HepG2, HuH-7 and TFK-1 cell lines, in which Everolimus seems to be particularly effective. Only in EGI-1, despite the use of high concentrations, mTOR inhibitors are effective up to 6 days of treatment. Prolonged treatment with all three mTOR inhibitors seems to trigger resistance in EGI-1. Moreover, we also found that octreotide has not synergistic or additive effect with rapamycin at high concentration, but when *in vitro* octreotide is used in combination with rapamycin at low concentration is able to revert the antiproliferative effect compared with rapamycin treatment in HepG2 and HuH-7, and this effects is also correlated to an iperexpression of somatostatin receptors subtypes 2 and 5 and upregulation of pMAPK, the proteins involved in survival pathways. To test the effect of another post-stimulus reaction,

AFP (α-fetoprotein), the actually accepted tumor biomarker for HCC, was tested. Corresponding to evidences of literature, after binding its receptor, SST activates an intracellular signalling directed to inhibition of hormonal secretion and probably, depending by downregulation of Ca^{2+} and cAMP intracellular level.

In conclusion, our data provide evidence of somatostatin receptor expression in HCC although a further characterization of the mechanism of SSTRs in the inhibition of HCC proliferation and secretion will be required to justify the clinical use of these compounds in the treatment of advanced HCC. So summarizing briefly, we think that SST and its analogues, after binding membranous receptors, activates an intracellular signalling that inhibits hormonal secretion downregulating AC, cAMP and Ca^{2+}; and cellular proliferation through the MAPK regulation. These intracellular mechanisms in HepG2 are mostly mediated by sstr2 and sstr5, while in HuH-7 also by sstr1. Somatostatin receptors do not have a main role in cholangiocarcinoma growth. mTOR inhibitors, instead, have an essentially growth inhibitory effect especially in HCC cell line, even though *in vitro* the rapamycin effect can be reverted by octreotide, showing that HCCs do not act as a typical neuroendocrine tumor.

Patients and methods

Samples of hepatocellular carcinomas

Twenty-three cases of advanced and unresectable HCC, diagnosed either by histology or by typical biochemical marker, alpha-fetoprotein (AFP) levels, were enrolled and considered for the study. After obtaining informed consent signed from all patients, the samples were achieved by liver biopsy of one nodule of HCC. The portions of HCC samples were immediately frozen in dry ice after the biopsy and stored in a freezer at -80°C until the performance of the molecular study.

Cell lines and culture conditions.

Two hepatocellular carcinoma cell lines, HepG2 cells and HuH-7, were obtained from European Collection of Cell Culture (ECACC) and from Health Science Research Resources Bank (HSRRB), respectively. HepG2 were cultured in RPMI 1640 medium with 10% of FBS, $1x10^5$ U/l penicillin and 2 mmol/l L-glutamine, HuH-7 were cultured in DMEM Glutamax medium with 10% of FBS and 1x105U/L penicillin and streptomycin; both cell lines were grown in a humidified condition in 5% CO_2 at 37°C. The human extrahepatic cholangiocarcinoma cell lines EGI-1 and TFK-1 were purchased from the German Collection of Microorganisms and cell Cultures (DSMZ) (Braunschweig, Germany). EGI-1 and TFK-1 were cultured in Dulbecco's Modified Eagle's Medium (DMEM) and RPMI 1640 respectively and supplemented with 10% of FBS (Gibco, Invitrogen, Naples, Italy) and penicillin 5000U/mL (Gibco, Invitrogen, Naples, Italy). The cell lines were grown in 75 cm^2 plastic flasks in humidified atmosphere of 5% CO_2 at 37°C.

Compounds

To carry out the proliferation and secretion assays, Somatostatin-14 (SS-14) was provided by Sigma Aldrich, Octreotide acetate was used as a stock solution of 100μg/ml (10^{-4}M) in acetate buffer; Pasireotide (SOM230), able to bind multiple somatostatin receptor subtype, was kindly supplied by Novartis Pharma AG (Basel, Switzerland). Lyophilized SOM230 was stored at 4°C and was reconstituted in sterile deionized water just before use at the necessary concentrations. Bim 23244 (bi-specific sstr2/sstr5 compound), Bim 23206 (with affinity for sstr5), Bim 23926 (selective for sstr1), Bim 23779 (universal ligand) were kindly supplied by M. Culler from Ipsen. Their respective affinities (Ki) are listed in *Table 1.2.* Rapamycin, Everolimus (Rad001) and Temsirolimus (CCI-779) were supplied by LC Laboratories (Inc. Woburn, MA, USA).

Ligands	Sst1	Sst2	Sst3	Sst4	Sst5
SS-14	**1.9**	**0.2**	**1.2**	**1.7**	**1.4**
Octreotide	>10000	**2.0**	187	>10000	22
Pasireotide	**9.3**	**1.0**	**1.5**	>1000	**0.16**
Bim 23244	1020	**0.29**	133	1000	**0.67**
Bim 23926	**3.6**	>1000	>1000	833	788
Bim 23206	1152	166	1000	1618	**2.4**

Table 1.2: Human somatostatin receptor (SSTR)-binding affinities of the analogues used for functional assays in this study compared with the SS-14. The values represent the affinity values expressed as Ki. The values in bold are the highest affinity of each compound for the respective receptor subtype(s).

Quantitative PCR

mRNA was isolated from the tissues and from cell lines with the use of Dynabeads Oligo (dT)25 (Dynal AS, Oslo, Norway). The poly (A+) mRNA was eluted in H_2O (65°C) for two times and for two minutes and 20μl were used for cDNA synthesis in a Tris buffer (50

mM Tris–HCl (pH 8.3), 100 mM KCl, 4 mM DTT and 10 mM MgCl2) with 10 units RNase inhibitor, 2 units avian myeloblastosis virus Super Reverse Transcriptase, oligo dT (5ng/μl) and 1 mM of each deoxynucleotide triphosphate in a final volume of 40 ml. This was incubated for 1 h at 42˚C and the resulting cDNA was diluted fivefold in 160 ml sterile H_2O. cDNA was used for quantification of mRNA levels of all investigated genes: hypoxanthine phosphoribosyltransferase (HPRT), sst1, sst2, sst3, sst5, D2, IGF1 and IGF1R. The total reaction volume (12.5 μl) consisted of 5μl of cDNA and 7 μl of TaqMan Universal PCR Mastermix (Applied Biosystems, Branchburg, NJ, USA) with primers–probes in the following concentrations: HPRT, sst2, sst3 and sst5 500–500–100 nM, sst1, D2, IGF1 and IGF1R 300-300-200 nM, of forward primer, reverse primer and probe respectively. The primer and probe sequences are shown in *Table2.2*. All primers and probes were purchased from Sigma–Aldrich. Real-time quantitative PCR was performed in 96-well optical plates with the TaqMan Gold nuclease assay (Applied Biosystems) and the ABI Prism 7700 Sequence Detection System (Perkin–Elmer, Foster City, CA, USA). After two initial heating steps at 50˚C (2min) and 95˚C (10min), samples were subjected to 40 cycles of denaturation at 95˚C (15s) and annealing at 60˚C (60s). All samples were assayed in duplicate. Values were normalized against the expression of the housekeeping gene HPRT. Dilution curves were constructed to calculate PCR efficiencies (E) for every primer–probe set [62]. Efficiencies were: sst1 1.91, sst2 1.91, sst3 1.92, sst5 1.92, D2 1.94, IGF1 1.98, IGF1R 1.85 and HPRT 1.91. Being a no ideal system, the relative expression of genes were calculated using the comparative threshold method, $2^{-\Delta Ct}$, [63] after efficiency correction [64] of target and reference gene transcripts. To exclude genomic DNA contamination in the RNA, the cDNA reactions were also performed without reverse transcriptase and amplified with each primer pair. To exclude contamination of the PCR mixtures, the reactions were also performed in the absence of cDNA template, in parallel with cDNA samples.

Gene	Primers	Probes
Sst1	Forward 5'-TGAGTCAGCTGTCGGTCATC-3' Reverse 5'-ACACTGTAGGCACGGCTCTT-3'	5'-FAM-ACAGCTGCGCCAACCCCATC-TAMRA-3'
Sst2	Forward 5'-TCGGCCAAGTGGAGGAGAC-3' Reverse 5'-AGAGACTCCCCACACAGCCA-3'	5'-FAM-CCGGACGGCCAAGATGATCACC-TAMRA-3'
Sst3	Forward 5'-CTGGGTAACTCGCTGGTCATCTA-3' Reverse 5'-AGCGCCAGGTTGAGGATGTA-3'	5'-FAM-CGGCCAGCCCTTCAGTCACCAAC-TAMRA-3'
Sst5	Forward 5'-CATCCTCTCCTACGCCAACAG-3' Reverse 5'-GGAAGCTCTGGCGGAAGTT-3'	5'-FAM-CCCGTCCTCTACGGCTTCCTCTCTGA-TAMRA-3'
D2 (long + short isoform)	Forward 5'-GCCACTCAGATGCTCGCC-3' Reverse 5'-ATGTGTGTGATGAAGAAGGGCA-3'	5'-FAM-TTGTTCTCGGCGTGTTCATCATCTGC-TAMRA-3'
mTOR	Forward 5'-TGCTGCGTGTCTTCATGCAT -3' Reverse 5'-GGATTGCAGCCAGTAACTTGATAG -3'	5'-FAM- ACAGCCCAGGCCGCATTGTC-TAMRA-3'
4eBP1	Forward 5'-GGCGGCACGCTCTTCA -3' Reverse 5'-TCAGGAATTTCCGGTCATAGATG -3'	5'-FAM- ACCACCCCGGGAGGTACCAGGA-TAMRA-3'
p70S6K	Forward 5'-TGGAAGACACTGCCTGCTTTT -3' Reverse 5'-TGATCCCCTTTTGATGTAAATGC -3'	5'-FAM- CTTGGCAGAAATCTCCATGGCTTTGG-TAMRA-3'
HPRT	Forward 5'-TGCTTTCCTTGGTCAGGCAGTAT-3' Reverse 5'-TCAAATCCAACAAAGTCTGGCTTATATC-3'	5'-FAM-CAAGCTTGCGACCTTGACCATCTTTGGA-TAMRA-3'

Table 2.2: Primer and probe sequences used in this study for the qRT-PCR.

Immunocytochemical analysis

HepG2 and HuH-7 cells were grown on eight-well chamber slides (BD Falcon; BD Biosciences, Belgium) coated with poly-D-lysine (1mg/ml; Sigma- Aldrich, St. Louis, MO) overnight. In basal condition and after treatment with various somatostatin analogs, the cells were fixed with 4% of paraformaldehyde and 0.2% of picric acid in phosphate buffer pH 6.9 for 40 min at room temperature and permeabilizated in 50% and in 100% ice-cold methanol (3 + 3 min). After several washes, a solution of 30% of H_2O_2/PBS was added to each well to quench endogenous peroxidise and the chamber slide was put in the dark for 15 min. After 2 washes with TRIS/HCl/Tween 0.5% the cells were incubated for 60 min at room temperature with an sst5-specific primary antibody (kindly provided from Prof. S. Schultz) and overnight with an sst2A-specific primary antibody (SS-8000; Biotrend, Koln, Germany) diluted 1:50 and 1:20 in Normal Antibody Diluent (ScyTek, Logan, Utah, USA) respectively and then washed three times for 5 min each with TRIS/HCl/Tween 0.5%. The cells were then incubated for 30 min at room temperature in the dark with 2 drops of secondary antibody: Dextran coupled with peroxidase molecules and goat secondary antibody molecules against rabbit and mouse immunoglobulins (HRP Rabbit/Mouse, Dako Detection System, Dako, Glostrup, Danemark). After 3 washes a solution of diaminobenzidine tetrahydrochloride (DAB) and substrate was added on slides that were incubated for 10 min in the dark. After washes with water, nuclei were stained with hematoxilin and the slides were examined using microscope Nickon Eclipse E400.

Western Blot analysis

Cell extracts were prepared as followed: cell samples were washed twice with PBS and homogenized in NP40 lysis buffer (1% NP-40, 10% glycerol, 137mM NaCl, 20mM Tris pH7.6, 20mM NaF) with protease inhibitors and phenylmethylsulfonyl fluoride (PMSF) in ice for 30 min. The homogenate was centrifuged for 15 min, at 1200 x g and 4°C, and the supernatant was stored at -80°C until use. The protein concentrations were determined

photometrically with a bicinchoninic acid (BCA) Protein Assay Kit (Thermo Scientific, USA). After protein denaturation by heating to 95°C for 2 min, we used 40μg of total extracts for immunoblotting. Proteins from cell preparations were separated by 8% SDS-PAGE and then, electro-transferred onto a nitrocellulose membrane by means of voltage application of 100V for 1h in a TransBlot Amersham apparatus and then, subjected at blocking treatment for 30 minutes with 5% of milk. After that, the same nitrocellulose sheet was probed with primary antibodies specific for:

Sstr2a (ss-8000; Biotred Koln; Germany);

ERK2 (sc-1647, Santa Cruz Biotechnology Inc; Italy);

pERK1/2 (sc-7383, Santa Cruz Biotechnology Inc; Italy);

ß-actin (A4700; Sigma Aldrich; Italy).

Subsequently they were bound with peroxidise-conjugated secondary antibodies:

Mouse (goat antimouse IgG-HRP Santa Cruz Biotechnology; Italy sc-2005);

Rabbit (donkey antirabbit IgG-HRP Santa Cruz Biotechnology; Italy sc-2313) and a detected with an ECL system. After chemioluminescent reaction, the blot was exposed to X-film for autography.

Cell proliferation assay

After trypsinization, HepG2 and HuH-7cells were plated in 1 ml of complete culture medium in 24-well plates at a density of $3x10^4$ cells/well and $3.5x10^4$ cells/well respectively. The plates were then placed in incubator in 5% CO_2 at 37°C. After 24h, the complete medium was removed and 1 ml of medium supplemented with 1% of serum was added to each well and after 2 hours the test compound (OCT) was added at different concentrations (10^{-11} to 10^{-7} M). Quadruplicates of each treatment were performed. Plates were further incubated at 37°C and 5% CO_2. After 3 days of treatment, cells were harvested for DNA measurement. Measurement of total DNA contents, representative for the number of cells, was performed using the bisbenzimide fluorescent dye (Hoechst 33258)

(Boehring Diagnostics, La Jolla, CA), as previously described [65]. Data are expressed as percent of the control.

Propidium iodide staining

Apoptosis was evaluated by propidium iodide (PI) staining of cells followed by flow cytometry analysis. Briefly, after 72h of treatment with each drug, HepG2 cells were incubated for 30 min in a hypotonic solution containing 50 μg/ml PI at room temperature in the dark. The stained cells were analyzed, for relative DNA content, on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) At least 10 000 events were counted for each sample. Debris was excluded by scatter gating. Cells with subdiploid DNA content (subG0/G1 peak) were considered apoptotic cells.

AFP assay

HepG2 cells ($3x10^4$ cells/well) and HuH-7 ($3.5x10^4$ cells/well) were cultured in 24-well plates and placed in incubator at 37°C and in 5% CO_2. After 24h, the cells were starved with medium supplemented with 1% of serum for 2 hours and then the test compound (OCT) was added in the range of 10^{-11} to 10^{-7} M. For controls, cells were placed in medium only. Each concentration was performed in quadruplicate wells. Plates were further incubated at 37°C and 5% CO_2 for 3 days. The amount of α-fetoprotein (AFP) in the culture supernatant was analyzed by immuno-chemiluminescent assay (CLIA) (Diasorin, Torino, Italia) consistingin two-step sandwich chemiluminescence immunoassay, using directly coated magnetic particles (as solid phase) and an isoluminol derivative (as conjugate).

Statistical analysis

Statistical analysis was performed using SPSS for Windows version 17.0 (SPSS Inc., Chicago, IL). Receptor expression levels in patients and in cell lines are expressed as mean ± S.E.M. Correlation analysis between the investigated genes were performed by Spearman

nonparametric correlation. Pearson correlation and linear regression were used to analyze the association between the genes and the clinical parameters. Results were presented as mean ± S.E.M. for cell proliferation and hormone assays experiments. Differences between treatment groups compared with control for in vitro data were assessed by ANOVA with a Bonferroni correction.

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Publications

1. *"The GH-IGF system in amyotrophic lateral sclerosis: correlations between pituitary GH secretion capacity, insulin-like growth factors and clinical features"*
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5. *"Dopamine receptor expression and dopamine agonist effectiveness in corticotroph pituitary tumors: comparison with clinical, biochemical, radiological and pathological featurs of patients with Cushing's disease"*
   Pivonello R, de Herder WW, Ferone D, Waaijers M, KROS JM, Del Basso De Caro ML, Cozzolino A, **Pivonello C**, Colao A, Hofland LJ, Lamberts LWJ . (In press on Journal of Clinical Endocrinology and Metabolism)

6. *"Dopamine D2 receptor expression in the corticotroph cells of the human normal pituitary gland".*
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