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Involvement of Interleukin-6 Induced PI3K/Akt/mTor Pathway in the Regulation of Telomerase and Alpha-Fetoprotein Expression in Hepatocellular Carcinoma

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Keywords:

Hepatocellular carcinoma; Télomérase; Alphafeotoprotein; Interleukin6

Abbreviations:

AFP: Alpha Fetoprotein; ATCC: American Type Cell Culture; BIBR: 2-[[(2E)-3-(2-Naphthalenyl)-1-Oxo-2-Butenyl1-Yl]Amino]Benzoic Acid; CCAs: Biliary Tract Epithelial Cancers; CH: Chronic Hepatitis; DNA: Deoxyribonucleic Acid; FBS: Fetal Bovine Serum; HBsAg: Antigen of Hepatitis B; HCC: Hepatocellular Carcinoma; IL-6: Interleukin-6; LC: Liver Cirrhosis; LPS: Lipopolysaccharide; MMPs: Metalloproteinases; Ncbp: Nuclear Cap-Binding Protein; NEAA: Non Essential Amino Acids; NK: Natural killers; P/S: Penicillin / Streptomycin; PI3K: Phosphoinositide 3- Kinase; PIP2: Phosphatidylinositol 4,5-Bisphosphate; PMA: Phorbol Ester; PTEN: Phosphatase and Tensin Homolog Deleted from Chromosome 10; RTK: Receptor Tyrosine Kinases; S6K: S6 Kinase Proteins; TAM: Tumor Associated Macrophages; TSC2: Tuberous Sclerosis Complex 2

1. Abstract

1.1. Background: Hepatocellular Carcinoma (HCC) is an invasive cancer. Alphafoetoprotein (AFP) is a diagnostic marker for HCC directly related to the disease agressivity. Télomérase, is expressed by 90% of HCC. PI3K/Akt/mTOR pathway wich is regulated by IL-6 is activated in the HCC. Our aim is to investigate the effect of IL-6 on AFP and telomerase secretion in HepG2/C3A and PLC/PRF/5 cell lines.

1.2. Material and Methods: Cells were cultured in complete DMEM, and incubated at 37o with 5% CO2. The viability test was performed using tetrazolium salt. The concentration of AFP was measured by ELISA kits. AFP mRNA level was measured using RT-PCR technique. PI3K, Akt, mTOR protein expression was assessed by western blot.The effect of telomerase inhibition on C/EBP was assessed by gel shift assay.

1.3. Results: Inhibition of PI3K/Akt/mTOR signaling pathway decreased AFP concentration suggesting the involvement of this pathway in AFP regulation. Decreasing in AFP concentration was shown after treatment with PKC inhibitor.IL-6 (25ng/ml) treatment showed a transitional increasing of AFP production after 8h and a decreasing effect after 10h, suggesting the involvement of IL-6 in AFP production modulation. Inhibition of telomerase decreased the phosphorylated form of PI3K, mTOR and STAT3 protein expression but not the binding of C/EBP promoter to AFP.

1.4. Conclusion: AFP and telomerase secretion is regulated by IL-6 via the PI3K/Akt/mTOR pathway in HepG2/C3A and PLC/PRF/5 cells.

2. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide with a global increasing annual incidence [1]. Many promising tumor markers for HCC have been recognized, such as des-gamma-carboxy prothrombin [2], lens culinaris agglutinin-reactive AFP [3], pancreatitis associated protein10 [4] and insulin-like growth factor-2 [5], but none of these

markers has been validated for clinical use. Although it is not the ideal one, the AFP remains the most important tumor marker for HCC is human embryonic-fetoprotein (AFP) [6]. The A-Fetoprotein (AFP) is a 70 kDa oncofetal glycoprotein composed of the 590 amino acid polypeptide and a carbohydrate moiety, and it is detected both fetally and maternally during pregnancy [7]. However, in HCC serum patients, the AFP level is significantly increased, and this protein is directly related to the aggressivity of the disease [8].

Telomerase is a ribonucleoprotein reverse transcriptase enzyme complex that serves to elongate the telomeric ends and compensates for the progressive shortening associated with DNA replication during each cycle of cell division. Telomerase is also known to be involved in HCC: somatic mutations in the TERT promoter are the most frequent genetic alterations in human HCC [9]. Moreover, it was reported that telomerase was significantly reactivated in HCC and other types of chronic liver diseases. However, telomerase activity in Chronic Hepatitis (CH) and Liver Cirrhosis (LC) is significantly lower than that in atypical adenomatous hyperplasia and HCC [10, 11].

In addition to AFP and hTERT upregulation in HCC, PI3K pathway is also activated in this type of cancer [12]. In fact, the family of lipid kinases named phosphoinositide 3- kinases (PI3Ks) phosphorylates phosphoinositides at the 3′-hydroxyl group [13]. The PI3Ks play key regulatory roles in many cellular processes including cell proliferation, survival, and differentiation [14]. They are activated by Receptor Tyrosine Kinases (RTKs) and their primary role is to convert PIP2 to PIP3, which in turn activate the serine/threonine kinase AKT and other downstream effector pathways [15] mTOR is a key protein in the pathway that acts both upstream and downstream of Akt. Akt directly phosphorylates and activates mTOR, as well as cause indirect activation of mTOR by phosphorylating and inactivating TSC2 (tuberous sclerosis complex 2, also called tuberin) [16]. When activated, mTOR regulates the synthesis of different proteins involved in cell growth, angiogenesis, proliferation and other cellular endpoints such as S6 kinase proteins (S6K1 and SK2) [17] and the nuclear Cap-Binding Protein (nCBP) [18].The most important negative regulator of the PI3K signaling pathway is the tumor suppressor PTEN (phosphatase and tensin homolog deleted from chromosome 10) [19]. Recently, studies have showed that many proteins of the PI3K pathway are frequently targeted by somatic or germline mutations in a large number of human cancers [20–22]. These mutations can confer tumourigenic caracteristics in several cellular systems making the PI3K/Akt/mTOR pathway one of the most attractive targets for therapeutic strategies in cancer [23]. We previously reported that telomerase inhibition decreases AFP production in HCC cell lines. Also, we have demonstrated that this relationship between AFP and telomerase could be via the PI3k/Akt/mtor signaling pathway [24].

In this study, we examined the relationship between telomerase, AFP and the pro-inflammatory cytokine IL-6 due to the several reasons. First, hTERT mRNA and AFP mRNA are up-regulated in the multistep process of hepatocarcinogenesis [25]. Second, the PI3K/Akt/mTOR signaling pathway that is thought to be involved in the regulation of AFP [26] and hTERT [27], is regulated by interleukin-6 [28]. In fact, IL-6 induces Akt/PKB phosphorylation in a time and dose dependent manner in multiple myeloma. The IL-6 induces the phosphorylation of downstream targets of Akt, including Bad, GSK-3β, and FKHR [29]. In addition, increased levels of interleukin (IL)-6 have been reported to associate with and have a negative survival impact on patients with various types of cancer including HCC. Moreover, IL-6 has been shown to link with some human cancers as well as HCC [30–32]. However, the potential role of IL-6, as tumor marker for HCC and its relationship with AFP is not fully clear.

3. Results

3.1. Dose-response curve effect of Interleukin-6 on AFP expression and secretion and on hTERT expression

In our study, we focused on evaluating the effect of interleukin-6 on the expression and secretion of AFP as well as the expression of hTERT. The figure 1 shows that treatment of HepG2 / C3A cells by IL-6 at different concentrations (2, 5 and 10 ng / ml) for 24 hours did not have any significant effect on the secretion of AFP in the cell supernatant. However, IL-6 (25ng / ml) significantly decreased the concentration of AFP in the supernatant of HepG2 / C3A cells. The same pattern was observed in PLC/PRF/5 cell line. This effect is also observed at the mRNA level of AFP (30% decrease after treatment with IL-6 at 25 ng / ml) in both cell lines. Moreover, IL-6 reduces the expression of hTERT at a concentration of 5 ng / ml by 20%). The effect was more pronounced at higher concentrations of IL-6, 10 and 25 ng / ml, (40 and 50%, respectively) in HepG2/ C3A cells while, in PLC/PRF/5 cells, IL-6 reduces the expression of hTERT only at a concentration of 25ng/ml by 30% (Figure 2).

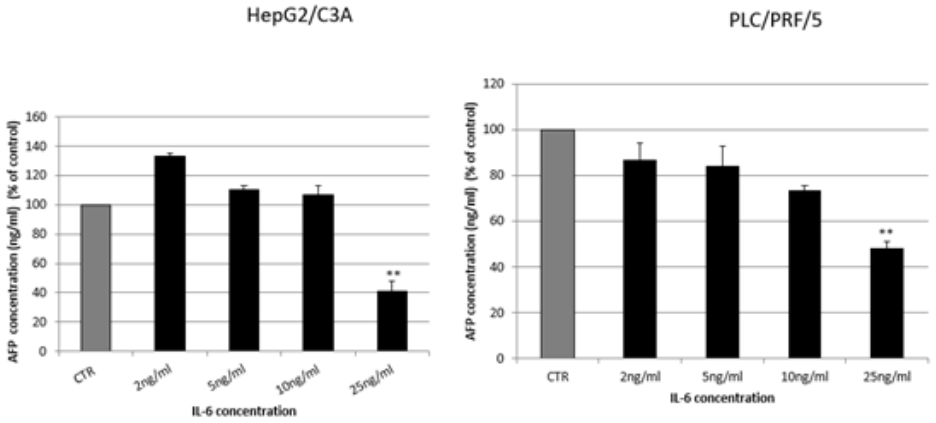


Figure 1: Effect of Interleukin-6 on AFP secretion in HepG2 / C3A and PLC/PRF/5 cells.
The cells are seeded in 6-well plates at a density of 2x105 cells / well. At 80% confluence, the cells were treated with interleukin-6 (2, 5, 10 or 25 ng / ml) for 24h. The supernatant was then collected and evaluated by ELISA. Each value represents the average of three experiments. Each value has its own control which was defined as 100%. Each point represents a mean + / _ SD for all experiments. ** P <0.005 compared to untreated cells.

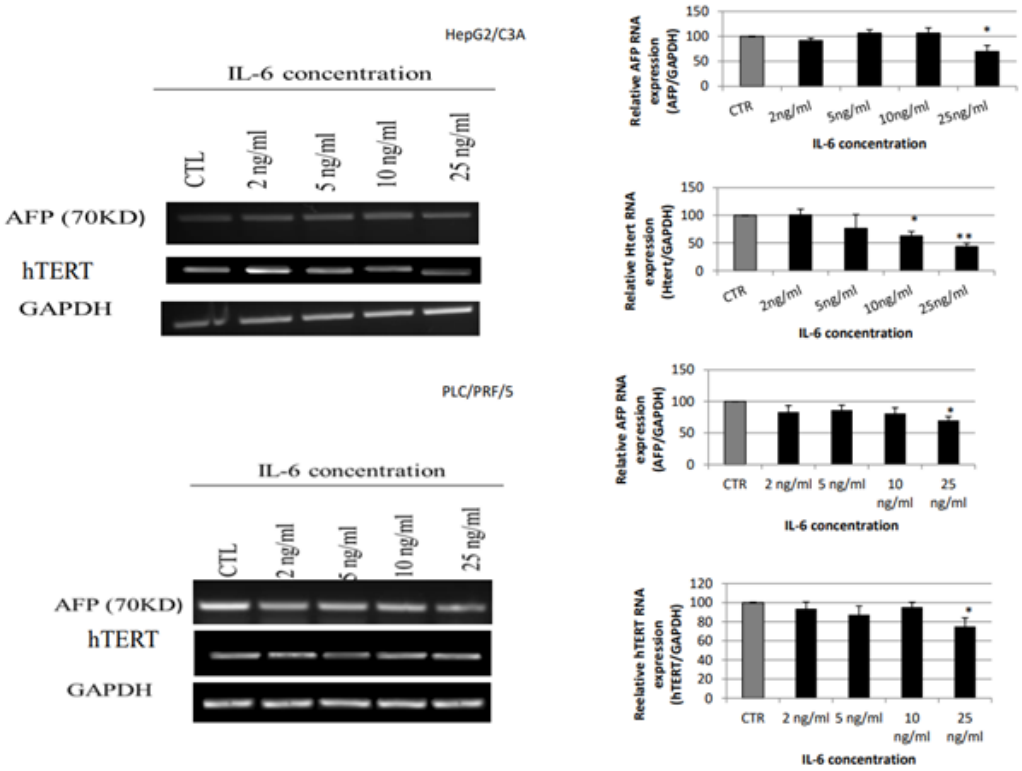


Figure 2: Effect of interleukin-6 on the mRNA expression of AFP and hTERT by HepG2 / C3A and PLC/PRF/5cells. Cells were seeded in 75 cm2 flasks in DMEM 1g / L. At 80% confluence, the cells were treated with interleukin-6 (2, 5,10 or 25 ng / ml) for 24h. RNA was extracted from cells for RT-PCR. The amplified DNA was then loaded on an 2% agarose gel and visualized by sybersafe staining. The relative mRNA expression was obtained by using the software Gelanalyzer.

3.2. Time course effect of interleukin-6 on AFP secretion and expression

Since IL-6 (25 ng / ml) significantly decreased the concentration of AFP in the supernatant of HepG2 / C3A cells after 24 hours of treatment, we have established a curve in time showing the effect of IL-6 (25 ng / ml) after various periods of treatment . This experiment gave us a clearer idea of the regulation of AFP by IL-6. Indeed, the figure 3 shows that IL-6 (25ng / ml) significantly reduces the concentration of AFP in the cell supernatant after 4 hours of treatment in HepG2/C3A cells and after 2 hours in PLC/PRF/5 cells. However, at 8 hours of treatment, this concentration increases significantly before falling again after 24 hours in both cell lines. This profile is also observed at the mRNA level of AFP. However, the transient effect of IL-6 on hTERT is less clear (Figure 4). Indeed, in HepG2/C3A cells, the expression of the hTERT was reduced by 20 and 25% after treatment with IL-6 for 2 and 4 hours respectively. After 6 hours of treatment, the expression of hTERT is comparable to that in the control, then after 8 hours of treatment, its expression increases to 20% prior to of its decrease by 30 to 50% after treatment for 10 and 24 hours respectively. While in PLC/PRF/5 cells, the expression of the hTERT was reduced by 30% after treatment with IL-6 for 2 hours. Then after 8 hours of treatment, the expression increases to 20%. After 10 hours of treatment, hTERT expression is comparable to that in the control well, while after 24 hours of treatment, the hTERT expression decreases by 40%.

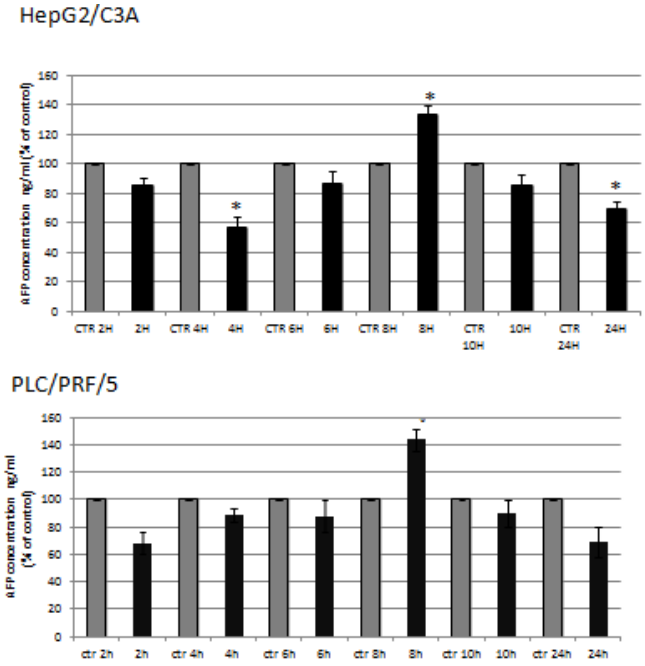
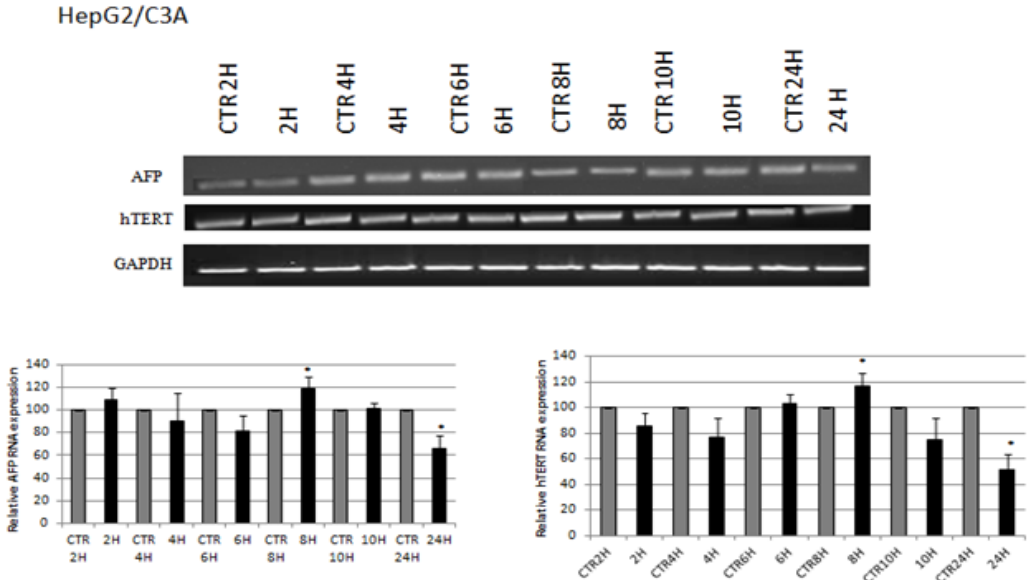


Figure 3: Time course effect of interleukin-6 on the AFP secretion.

The cells are seeded in 6-well plates at a density of 2x105 cells / well. At 80% confluence, the cells were treated with interleukin-6 at 25 ng / ml for 2, 4, 6.8 10 or 24. The supernatant was then collected and evaluated by ELISA. Each value represents the average of three experiments. Each value has its own control which was defined as 100%. Each point represents a mean + / _ SD for all experiments. ** P <0.005 compared to untreated cells.



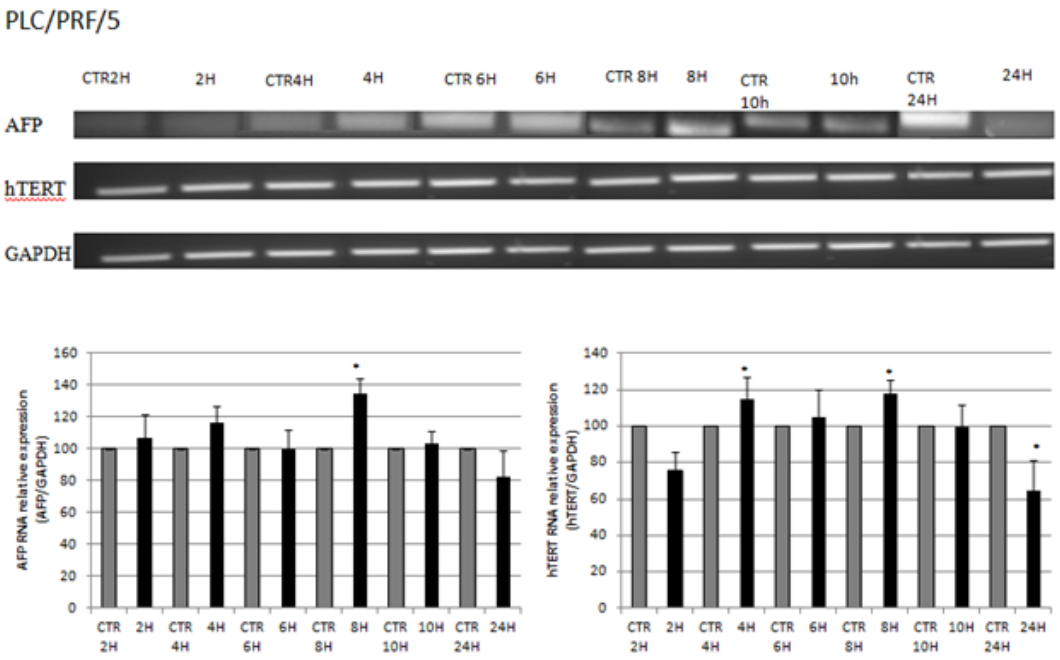


Figure 4: Effect of interleukin-6 on the mRNA expression of AFP and hTERT in HepG2 / C3A and PLC/PRF/5 cells.Cells were seeded in 75 cm2 flasks in DMEM 1g / L. At 80% confluence, the cells were treated with interleukin-6 at 25 ng / ml for 2, 4,6,8, 10 or 24h. RNA was extracted from cells for RT-PCR. The amplified DNA was then loaded on an agarose 2% gel and visualized by staining sybersafe. The relative mRNA expression was obtained by using the Gelanalyzer software.

3.3. Effect the PI3K / Akt / mTOR pathway inhibitors and IL-6 on the expression and the secretion of AFP and on the expresion of the hTERT

To assess the involvement of the PI3K / Akt / mTOR pathway in the relationship between AFP, IL-6 and telomerase, we proceed to study the effect of inhibitors of this pathway and the effect inhibitors of telomerase on AFP in HepG2 / C3A and PLC/PRF/5 cells. As shown in figure 5, the inhibition of PI3K, Akt and mTOR by wortmanin (5 nM), niclosamide (31 nM) and rapamycin (200 nm) for 8 hours, respectively, significantly decreased the concentration of AFP in the supernatant of HepG2 / C3A cells after one hour of treatment. This effect is also observed after 8 hours of treatment. The treatment of HepG2 / C3A and PLC/PRF/5 cells by interleukin-6 (25ng / ml) for 8 hours significantly increased the concentration of AFP in the cell supernatant (70%). However, the combination of pathway inhibitors PI3K/Akt/mTOR with IL-6 decreased the concentration of AFP in the supernatant suggesting that the regulation of AFP by IL-6 passes through this signaling pathway. LPS (lipopolysaccharide), an activator of telomerase did not have a significant effect on the concentration of AFP. However, inhibition of telomerase by the costunolide (10uM) and BIBR1532 (10uM) significantly decreased the concentration of AFP by 50 and 70% respectively in HepG2/C3A cells and by 40% and 30% in PLC/PRF/5 cells . The combination of telomerase inhibitors with IL-6 further increased the concentration of AFP. The relationship between AFP and PKC was also studied in this experiment, since PKC is known to be overexpressed in cancers and regulated by telomerase. Indeed, activation of PKC by PMA (phorbol ester) significantly increased the concentration of AFP by 60% and 70% in HepG2/C3A and PLC/PRF/5 cells respectively. The combination of PMA and IL-6 reinforced this effect and showed an additive effect / synergistic between the two molecules. Similarly, treatment of cells with the PKC inhibitor (SPC028) decreased the concentration of AFP in the supernatant (60% and 40% in HepG2/C3A and PLC/PRF/5 respectively, however the combination of IL-6 with the SPC028 restored this inhibition. The same pattern was observed with HepG2/C3A cells at the hTERT and the mRNA level of AFP except for PMA where the significance was not reached on the mRNA of hTERT in combination with IL-6 (25ng / ml) (Figure 6A). PLC/PRF/5 cells treatment with IL-6 (25ng/ml) increases AFP and hTERT expression after 8 hours (40 and 80% respectively), the expression of the two genes decreases when IL-6 was combined with PI3K, mTOR and STAT-3 inhibitors. Inhibition of telomerase by the costunolide (10uM) and BIBR1532 (10uM) significantly decreased the expression of AFP by 20 and 25% respectively. However, only BIBR1532 had a significant effect on the expression of hTERT (40%) . The combination of telomerase inhibitors with IL-6 increased once again the expression of AFP and hTERT. The PKC inhibitor had a significant effect on AFP expression but not on hTERT expression (Figure 6B).

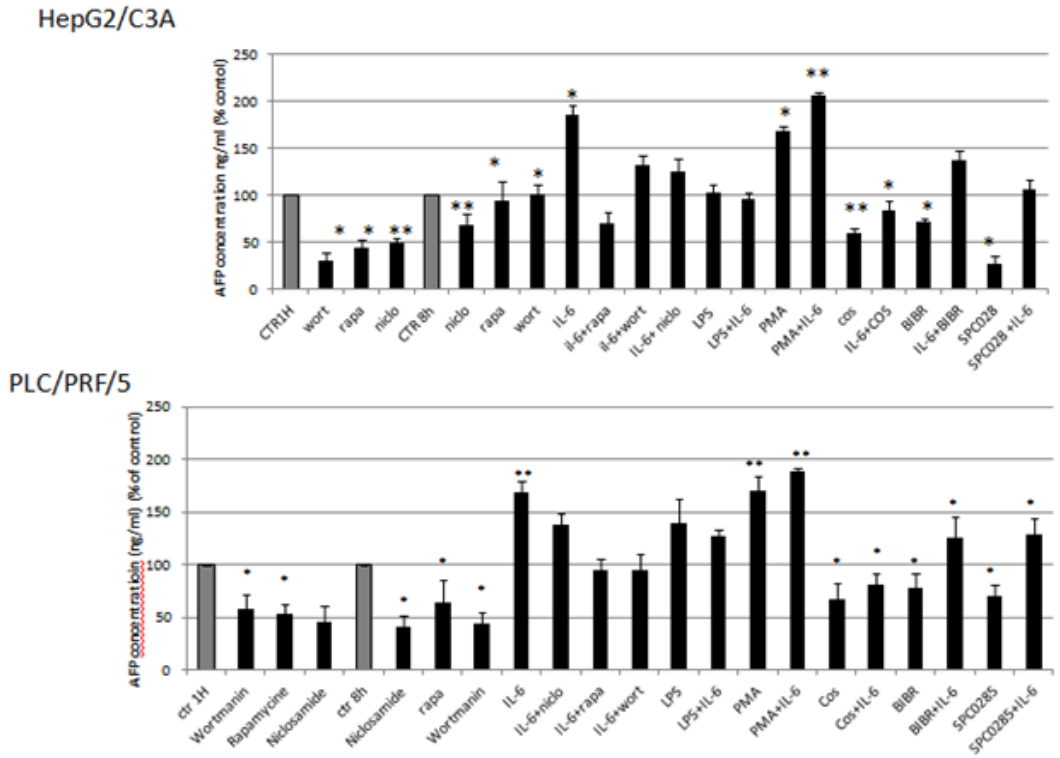


Figure 5: Effect of interleukin-6, inhibitors of PI3K / Akt / mTOR pathway and telomerase inhibitors on the secretion of AFP by HepG2 / C3A and PLC/PRF/5 cells.

The cells are seeded in 6-well plates at a density of 2x105 cells / well. At 80% confluence, the cells were treated by inhibitors of the PI3K / Akt / mTOR pathway for 1h. Then IL-6 was added with or without the inhibitors mentioned in the figure and the treatment of cells was carried out for 8 hours. The supernatant was then collected and evaluated by ELISA. Each value represents the average of three experiments. Each value has its own control which was defined as 100%. Each point represents a mean + / _ SD for all experiments. ** P <0.005 compared to untreated cells.

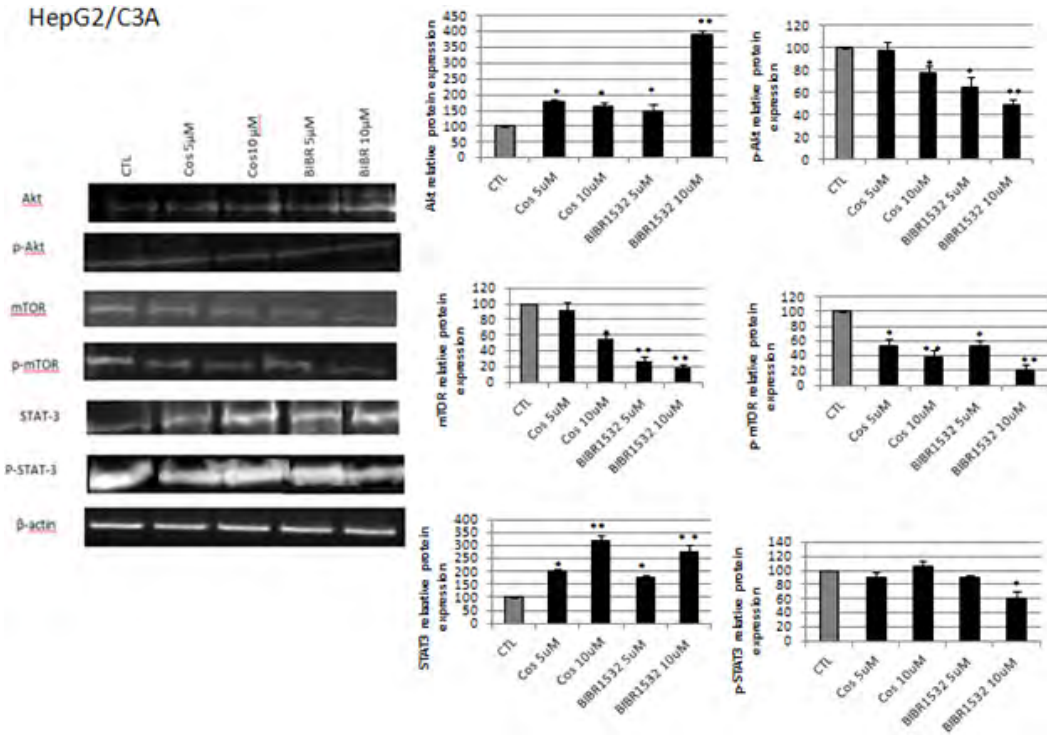


Figure 6: Effect of interleukin-6, inhibitors of the PI3K / Akt / mTOR pathway and telomerase inhibitors on the mRNA expression of AFP and hTERT in HepG2 / C3A and PLC/PRF/5 cells. The cells are seeded in 75 cm2 flasks in DMEM 1g / L. At confluence 80%, the cells were treated by inhibitors of the PI3K / Akt / mTOR pathway for 1h. Than IL-6 was added with or without the inhibitors mentioned in the figure and the treatment of cells was carried out for 8 hours. RNA was extracted from cells for RT-PCR. The amplified DNA was then loaded on an 2% agarose gel and visualized by staining sybersafe. The relative mRNA expression was obtained by using the software Gelanalyzer

3.4. Effect of telomerase inhibitors on the expression of the components of the PI3K / Akt / mTOR pathway

The PI3K / Akt / mTOR pathway is known to regulate the expression of hTERT and telomerase activity [33,34]. The aim of this experiment was to explore the interaction between telomerase and the PI3K / Akt / mTOR pathway (Figure 7). A shows that the inhibition of telomerase by costunolide and BIBR1532 (5 and 10uM) increases the secretion of the non-phosphorylated form of Akt protein and STAT-3 in HepG2 / C3A cells, whereas only costunolide (10uM) and BIBR1532 (5 and 10uM) decreased the secretion of the phosphorylated form of the two proteins. Moreover, the secretion of the two forms, phosphorylated and non-phosphorylated, of the mTOR protein decreased significantly after treatment with the telomerase inhibitors at 5 and 10uM. However, the effect of telomerase inhibitors was slightly different in PLC/PRF/5 cells (Figure 7B). In fact, only costunolide at 10uM increases the secretion of the non-phosphorylated form of Akt protein and STAT-3, while BIBR1532 (5uM) increases the secretion of the non-phosphorylated form STAT-3 protein. Costunolide and BIBR1532 (5 and 10uM) decreased the secretion of the phosphorylated form of the two proteins. Moreover, the secretion of the two forms, phosphorylated and non-phosphorylated, of the mTOR protein decreased significantly after treatment with costunolide (5uM).

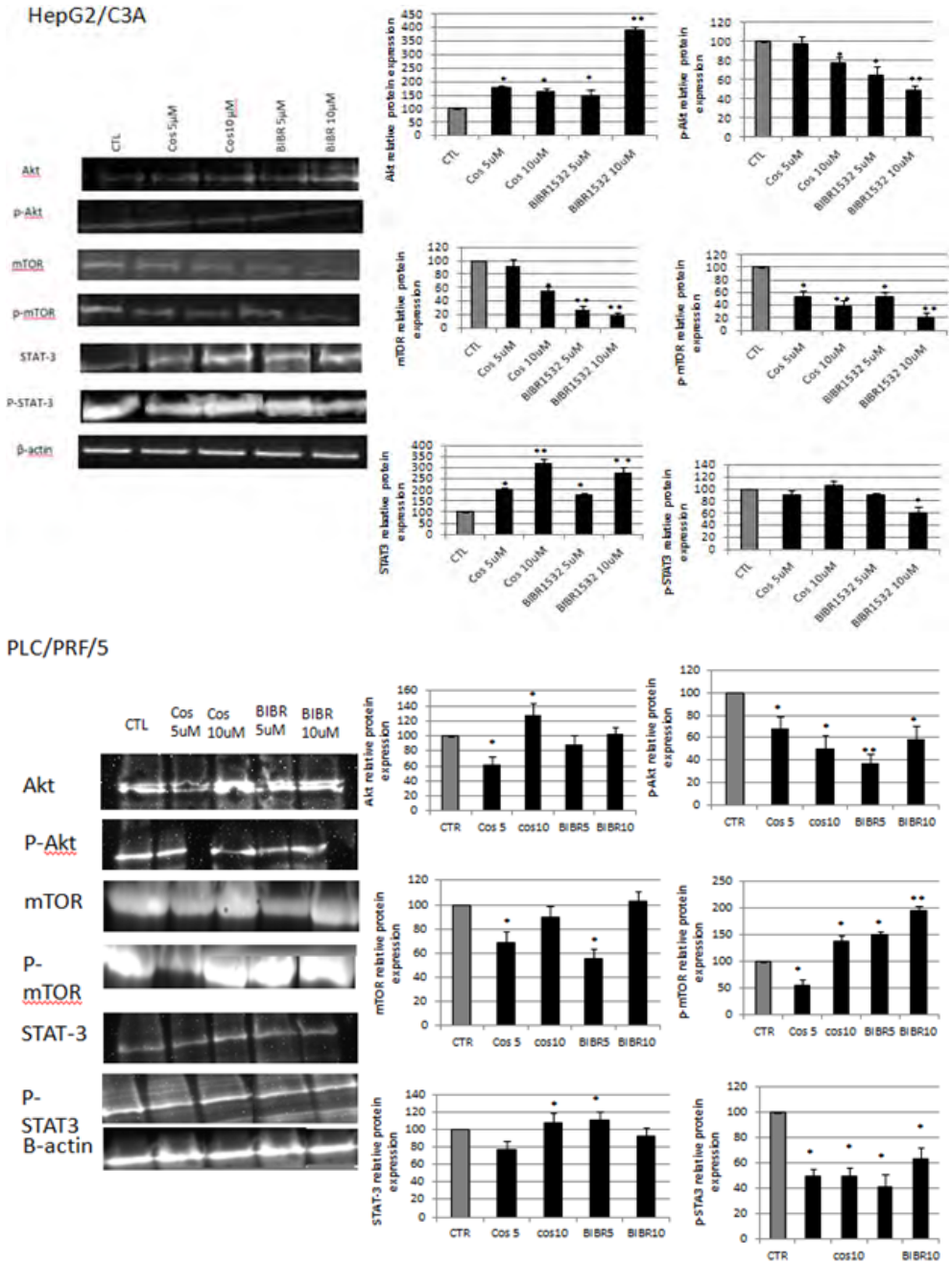


Figure 7: Effect of telomerase inhibitors of the PI3K / Akt / mTOR pathway in HepG2 / C3A and PLC/PRF/5 cells. Cells are seeded in 75cm2 flasks in DMEM containing 1g / L glucose. At 80% confluence, the HepG2 / C3A and PLC/PRF/5 cells are treated with the costunolide (5 or 10um) or BIBR1532 (5or 10um) for 48 h. Proteins were extracted with a lysis buffer and a Western blot was performed as described in the "materials and methods". The relative expression of the protein compared with the expression of B-actin is obtained using the Gelanalyzer software.

3.5. Effect of interleukin, inhibitors of the PI3K / Akt / mTOR pathway and telomerase inhibitors on telomerase activity

To explore the effect of IL-6 as well as the effect of telomerase inhibitors and the PI3K / Akt / mTOR pathway inhibitors in HepG2 / C3A and PLC/PRF/5 cells, the measurement of telomerase activity was performed. As shown in Figure, IL-6 (25ng / ml) activates telomerase whereas the inhibitors of the PI3K / Akt / mTOR (Wortmanin, Rapamycin and Niclosamide) show an inhibition effect after 8h. Inhibitors combining with IL-6, it is noted that activation of IL-6 is lifted in comparison with IL-6 alone. In addition, the treatment of cells with telomerase inhibitors, costunolide and BIBR 1532 and with the PKC inhibitor SPC028, decreases the telomerase activity in a very significant way (Figure 8).

3.6. Investigation of the relation between AFP and telomerase by EMSA

Several transcription factors have been reported to be involved in the regulation of the AFP gene, including Nkx2.8, HNF1, CEBP, NF1, RAR and RXR. Since CEBP is a common transcription factor between AFP and telomerase, our goal is to evaluate the effect of telomerase inhibitors on CEBP by gel shift. As shown in figure 9, BIBR1532 did not have an effect on CEBP binding to AFP promoter in HepG2/C3A and PLC/PRF/5 cells. Surprisingly, treatment with costunolide (10uM) for 48 hours induced the binding of CEBP to AFP promoter in both cell lines.

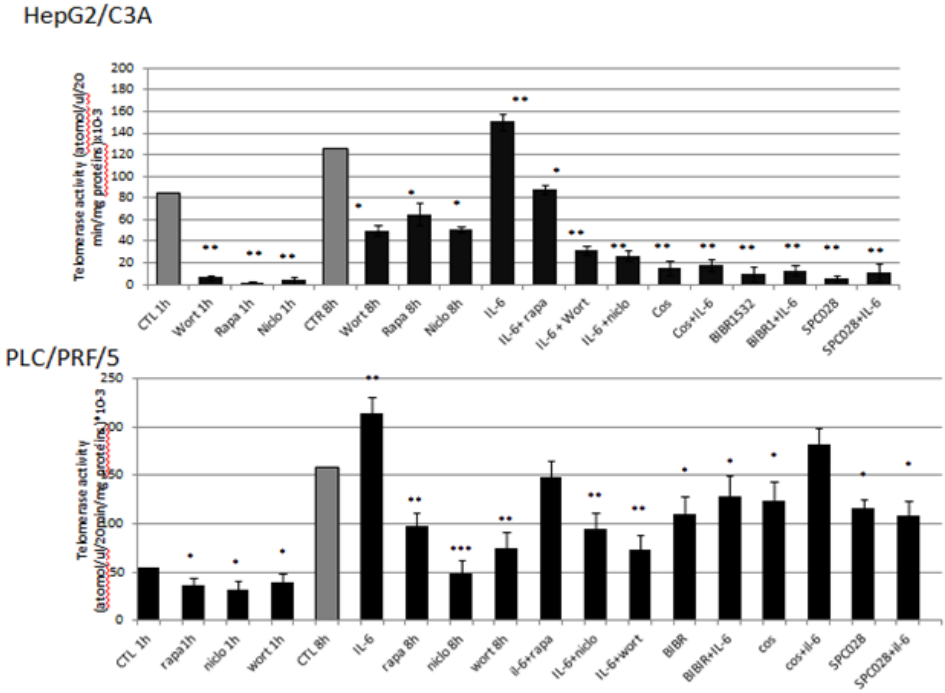


Figure 8: Effect of IL-6 inhibitors and telomerase inhibitors of the PI3K / Akt / mTOR on telomerase activity. Cells are seeded in 6 well plates in DMEM 1g/L. At 80% confluence, cells are treated with inhibitors of the PI3K / Akt / mTOR pathway, telomerase inhibitors and IL-6 (25ng/L). Proteins were extracted with a lysis buffer then a qPCR was performed. Each value represents the average of three experiments. Each point represents a mean + / _ SD for all experiments. ** P <0.005 compared to untreated cells.

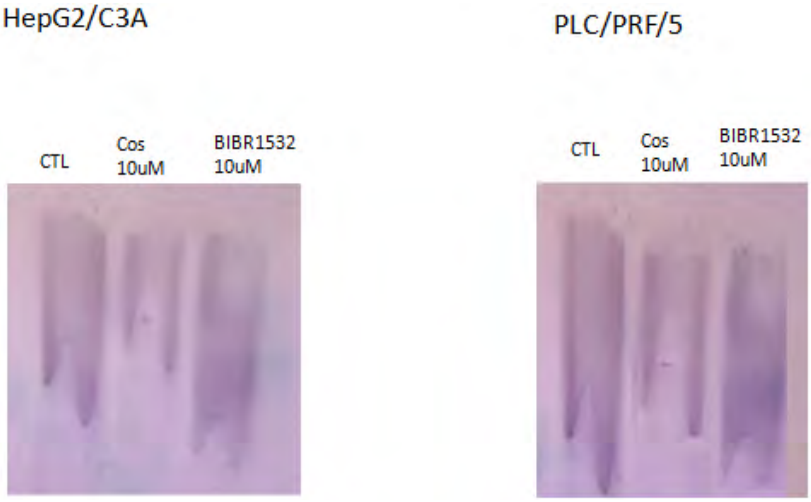


Figure 9: Effect of telomerase inhibitors on CEBP. Cells are seeded in 6 well plates in DMEM 1g/L. At 80% confluence, cells are treated with BIBR1532 and costunolide at 10µM for 48h. Proteins were extracted with a lysis buffer and a gel shift was performed as described in the "materials and methods".

4. Discussion

Telomerase activity is an important element in malignant transformation [35]. This study shows that telomerase inhibition decreased AFP secretion and expression in hepatocellular cell lines via the PI3K/Akt/mTOR, the anabolic pathway that regulate the telomerase activity.. In addition to an enhanced telomerase activity, malignant tumors are characterized by aberrant growth signaling mechanisms that permit escape from physiological controls of cell growth [36,37]. The major findings of our studies are that IL-6, a biliary epithelial mitogen, increases telomerase activity and AFP expression in HepG2/C3A and PLC/PRF/5 cells, involving the activation of PI3K/Akt/mTOR pathway. Thus, IL-6 stimulation may contribute to hepatocellular growth by increasing AFP secretion as well as promoting telomerase activity. Moreover, we demonstrated that the transcription factor C/EBP is not involved in this interrelationship between AFP and telomerase.

IL-6 has been implicated, as an autocrine promoter, of several types of cancer such as CCAs (biliary tract epithelial cancers) [38], multiple myeloma [39] and prostate cancer growth [40]. Thus, the regulation of telomerase expression by this cytokine may represent a unique mechanism resulting in tumor growth and progression. Our results support the presence of a dominant IL-6 stimulated pathway that increases telomerase expression and activity through the PI3K/Akt/mTOR pathway. Moreover, this pathway may be implicated in AFP regulation in hepatocellular carcinoma cells. In fact, it was demonstrated, in this study, that IL-6 increased AFP secretion and expression after 8h of treatment in a dose-dependant way. Potapovich et al. have showed that AFP may modulate inflammatory events in human keratinocytes by de novo expression of a number of pro-inflammatory mediators and modulation of their pro-inflammatory response to cytokines including IL-6 [41]. Recently, Zheng et al. have shown that AFP increases proliferation of hepatocellular carcinoma cells by stimulating PI3K / Akt / mTOR pathway. In fact, comparing with cirrhosis patients and control group, patients suffering from HCC, have serum level of AFP considerably increasing as the mRNA expression of PI3K and Akt. In addition, after incubation of HepG2 cells (which strongly express AFP) with monoclonal antibodies or AFP inhibitors, the expression of PI3K and Akt was significantly decreased [42]. These results point in the same direction as the results found by ZHU Zhi-Li and his colleagues have studied the correlation between PI3K, AFP and phosphorylated Akt (p-Akt). Indeed, a positive correlation was found between the three effectors. Additionally, this team showed that the expression of PI3K, p-Akt and AFP is very important in the growth of hepatocellular carcinoma and the expression of AFP can activate the PI3K / Akt pathway [43]. In our study, we showed that IL-6 increased AFP secretion in cells supernatant after 8h of treatment, then, when PI3K/Akt/mTOR inhibitors were combined with IL-6, AFP secretion decreased. These results suggest that IL-6 regulates AFP secretion through PI3K/Akt/mTOR pathway in hepatocellular carcinoma, andshow that it could be a close link between inflammation and hepatocellular carcinoma. Indeed, several authors have focused on this relationship. Barashi et al. recently showed that in a mouse model with HCC and lacking the CCR5 chemokine receptor, recruitment of macrophages and traffic to the liver were significantly reduced. In addition, in the absence of CCR5, the mice showed a significant decrease in tumor size in the liver [44]. Moreover, Capece et al. stated in their review of the literature that Tumor Associated Macrophages (TAM) play a central role in the case of hepatocellular carcinoma. Indeed, the TAM activate the growth of hepatocellular carcinoma cells (HCC), angiogenesis, invasion and metastasis, as well as the suppression of anti-tumor immune response by interacting with stromal and cancer cells within the microenvironment of the tumor.TAM release of many cytokines, chemokines and growth factors, which are involved in tumorigenesis. In particular, IL-6 and TGF factor which promote tumor growth, whereas TNF-αand tumor Metalloproteinases (MMPs) are involved in invasion and metastasis [45].

In the last recent years, several authors have been interested in the study of signaling pathways, especially the PI3K / Akt / mTOR pathway, which regulate hTERT and the activity of telomerase. Mengsen et al. have shown that mTOR can regulate directly or indirectly the activity of telomerase in HCC cell. However, there is no report on the role of chemotherapeutic agents on mTOR and its role in regulating the expression of hTERT profiles. Their results suggest that treatment of the cell line HCC SMMC-7721 with 5-fluouracile could downregulate the secretion both mTOR and telomerase activity. In addition, inhibition of mTOR leads to a decrease in telomerase activity at post-transcriptional level [46]. In addition, a study by Bellon et al. showed that inhibition of PI3K / Akt pathway reduces telomerase activity 33. The relationship between hTERT and PI3K / Akt pathway has been studied by Kawaushi et al. This team has found that stimulation of human NK cells (natural killer) IL-2 induces activation of the hTERT and the mechanism of activation of the hTERT after stimulation by IL-2 involves transcriptional regulation or post-translational through the PI3K / Akt / mTOR pathway. All these studies have shown that inhibition of PI3K / Akt / mTOR inhibits the expression of the hTERT or telomerase activity [47]. In our study, the opposite effect was studied for the first time, namely the effect of inhibition of telomerase on the components of the PI3K / Akt / mTOR pathway. Our results clearly show that telomerase inhibition by costunolide BIBR 1532 leads decrease the secretion of the components of the PI3K / Akt / mTOR after a 48 hours treatment as well as telomerase activity in HepG2 / C3A cells and PLC / PRF / 5 cells of hepatocellular carcinoma. Finally, this study shows that CEBP, a critical regulator of several hepatic metabolic processes, was not implicated in the relationship between AFP and telomerase. In fact, telomerase inhibition by BIBR1532 had no effect on

CEBP binding to AFP. Further studies are needed to explore the mechanism by which telomerase regulate AFP in hepatocellular carcinoma cells.

5. Materiel and Methods

5.1. The choice of cell lines

HepG2 / C3A cells: HepG2 / C3A cells are a hepatocellular carcinoma cell line provided by Baylor College of Medicine, maintained by ATCC (American Type Cell Culture) in the United States. HepG2 / C3A strongly express telomerase and secrete almost all proteins normally found in normal serum and also the AFP. These cells are cultured in our laboratory as recommended by ATCC (American Type Cell Culture). Indeed, HepG2 / C3A are cultured in DMEM + 10% FBS (Fetal bovine serum) + 1% P / S (penicillin / streptomycin) + 1% NEAA (Non Essential amino acids) and incubated at 37 ° in an incubator at 5% CO_2 PLC / PRF / 5 cells: PLC / PRF / 5 cell line is another hepatocellular carcinoma maintained by the ATCC in the USA. PLC / PRF / 5 express telomerase and AFP. In addition, these cells also express the surface antigen of hepatitis B (HBsAg) .As HepG2 / C3A, PLC / PRF / 5 were grown in our laboratory according to the recommendations of ATCC. Indeed, PLC / PRF / 5 were grown in DMEM + 10% FBS (Fetal bovine serum) + 1% P / S (penicillin / streptomycin) and incubated at 37 ° in an incubator with 5% CO_2.

5.2. Measurement of human AFP in cell supernatant

The concentration of human AFP in the cell supernatant was measured using an ELISA kit from HUMAN Company (GmbH) according to the manufacturer's recommendations. Briefly, HepG2 / C3A and PLC / PRF / 5 cells were seeded in 75 cm^2 flasks in culture medium. At 80% confluence, the cells were treated as described in the figure legends. The supernatant was then collected and diluted 1:50 with serum free media for ELISA; however, the supernatant of PLC / PRF / 5 was not diluted. The ELISA method is based on the affinity of biotin for streptavidin attached to the surface of a microtiter well. The enzyme-antibody conjugate is mixed with samples and standards to form the sandwich complex. After incubation and washing, substrate is added and the formed product is evaluated by reading the optical density using an ELISA reader at 450nm. The intensity of the color developed by the product is directly proportional to the concentration of AFP in the samples.

5.3. Cell viability test

Cell viability was determined using the tetrazolium salt 3- (4,5-dimethylthiazol-2-yl) -5- (3-carboxymethoxyphenyl) -2- (4-sulfophenyl) -2H as reported by the manufacturer's instructions (Sigma-Aldrich). The tetrazolium salt is cleaved to formazan by succinate- reductase, an enzyme that exists only in the mitochondrial respiratory chain and is active only in viable cells. Formazan production is proportional to the number of living cells in culture. Briefly, HepG2 / C3A and PLC / PRF / 5 were seeded at 10^4 cells per well in 96 well plates. At 80% confluence, the cells were treated with IL-6, PI3K/Akt/mTOR or telomerase inhibitor for 48 h. After treatment, 10 ul of tetrazolium salt were added to 100 uL of culture medium, and the plate was incubated for 45 min at 37ºC. Absorbance was then measured using an ELISA reader at 450 nm.

5.4. RNA extraction and RT-PCR

Total RNA from HepG2 / C3A and PLC / PRF / 5 was extracted using the kit "GenElute Mammalian Total RNA Kit" from Sigma-Aldrich according to manufacturer's instructions. The cDNA was synthesized from 1 ug of RNA using the kit "iScript cDNA Synthesis" (Bio-Rad). Quantitative PCR was performed using the kit REDTaq Ready Mix PCR Reaction Mix (Sigma-Aldrich). The PCR primers have the following sequences:

AFP forward 5'-ACCCTGGTGTTGGCCAGT'-3'; AFP reverse 5'-GCAGCGCTACACCCTGAGT-3'; hTERT forward 5'-TGAACTTGCGGAAGACAGTGG -3 '; hTERT reverse 5'-ATGCGTGAAACCTGTACGCCT-3'; GAPDH forward 5'-TGGGATGGACTGTGGTCATGAG-3; GAPDH reverse 5'-ACTGGCGTCTTCACCACCATGG-3'

The DNA was amplified for 35 successive cycles (denaturation: 95 ° C, 30 sec; annealing of the primers: 60 ° C, 1 min and elongation 72 ° C, 5 min). The amplified DNA was then run in an agarose gel at 2% and visualized by staining with SYBR Safe using the system BioDoc UVP.

5.5. Western blot

Cells were seeded in 75cm2 flasks with DMEM culture medium). At 80% of confluence, cells were treated by costunolide (5 or 10uM) and BIBR1532 (5 or 10uM) for 48 hours and the IL-6 (5.10 or 25 ng / ml) for 8h and 24h. After treatment, proteins were extracted from the cells with a lysis buffer comprising the following compounds: 50 mM NaCl, 5 mM EDTA, Triton X-100 1% (detergent allowing the destructuring of membranes), 50 mM NaF, Na4P2O7 10 mM protease inhibitor cocktail Roche® (Complete ™ Mini Protease Inhibitor cocktail), pH 7.5 for 30 min at 4 ° C. Cell debris were then removed by centrifugation at 10 000g for 10 min at 4 ° C.Extracted proteins are then denatured by adding a denaturing solution (Lamelli buffer 2x) and 2 ul B-mercaptoethanol to a final volume of 20ul. The mixture is then incubated for 5 min at 95ºC. Once denatured, the proteins are separated on an SDS-PAGE denaturing acrylamide gel in which the percentage varies depending on the molecular weight of the protein to be detected.

The proteins in the gel were then transferred onto a PVDF membrane by applying a current of 360mA intensity for 1 h. The membrane is then immersed in a solution of 5% milk for 1 hour with stirring to saturate the sites of nonspecific interactions of the membrane. The primary antibody (akt, mTOR or STAT-3 under their phosphorylated or unphosphorylated form) was diluted and incubated with the membrane overnight. The next day, the membrane was rinsed with a wash solution, and then the secondary antibody coupled to peroxidase was added and incubated with the mem-

brane for one hour and a half. The membrane was then placed in the presence of reactive chimiolumininescent UptiLight blot HRP ™ substrate (Uptima®) prepared according to the manufacturer's protocol in the dark. After one minute of incubation, the revelation was made using the UVP Biodoc system.

5.6. Measurement of telomerase activity

To see the effect of IL-6 and inhibitors of the PI3K / Akt / mTOR on the telomerase activity of cells, we used the kit "Quantitive Telomerase Detection Kit" of Allied Biotech Company. Briefly, HepG2 / C3A and PLC/PRF/5 cells were seeded as mentioned above and then treated with PI3K, Akt and mTOR inhibitors for 1h. Then, IL-6 (25ng / ml) was added and the treatment was performed during 8h. After treatment, the cells were incubated 30min on ice with lysis buffer then centrifuged at 14000rpm at 4 ° C for 30 minutes to extract telomerase. A dilution series was then produced from a solution of oligonucleotides having the same sequence as that of telomers (TSR) and a master mix containing primers telomere is added to each tube. Next, quantitative PCR was performed using the following protocol (Table 1).

Table 1:

Steps	Time	Temperature	Comments
Telomerase Reaction	20 min	25oC	Telomere templates were formed by adding 6-base-repeates to primer with the activity of telomerase
PCR Initial Activation Step	10 min	95oC	HotActivitedTag DNA Polymerase is activated by this heating step
3-step cycling:			
Denaturation	30s	95oC	
Annealing	30s	60oC	
Extention	30s	72oC	
Cycle number	35-40 cycles		Cycle number depends on the amount of template DNA.

5.7. Gel shift assay

Single-stranded biotin-labeled oligonucleotides containing C/EBP consensus DNA binding site were purchased from Bio-Rad. Double-stranded oligonucleotides were prepared by annealing complementary oligonucleotides in a buffer containing 10 mM Tris (pH 8.0), 50 mM NaCl, and 1 mM EDTA. The sequence of the complementary pairs are as follow: AFP C/EBP, -118 to -97, 5'-CAAATTGCCTAACTTCAACATA-3', CEBP-BIOTEG-1 5'-TGCAGATTGAAGCAATTTCCTCCTGCA-3', CEBP 3' ACGTCTAACTTCGTTAAAGGAGGACGT-5'. For the EMSA, labeled double-stranded oligonucleotides were incubated with nuclear cell extracts in a binding buffer containing 10 mM Tris (pH 8.0), 1mM EDTA, 50 mM NaCl, BSA (1.45 mg/ml), glycerol and poly d(I-C) (1 µg/µl).

5.8. Statistical Analysis

Results were assessed by t-test using the Graph-Pad Quickcalcs online software (http://www.graphpad.com/quickcalcs/ttest1.cfm). Values are expressed as means SD.

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