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EPIGENETIC MODIFICATIONS IN HEPATOCELLULAR CARCINOMA. CAN EPIGENETIC MODULATING DRUGS PLAY A ROLE ON HEPATOCELLULAR CARCINOMA THERAPEUTICS?

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EPIGENETIC MODIFICATIONS IN HEPATOCELLULAR CARCINOMA. CAN EPIGENETIC MODULATING DRUGS PLAY A ROLE ON HEPATOCELLULAR CARCINOMA THERAPEUTICS?

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Index

RESUMO

O Carcinoma hepatocelular é a segunda causa mais frequente de mortes relacionadas com o cancro, com prevalência mais elevada no Sudeste Asiático e nos países africanos devido a taxas elevadas de infecção crónica pelo vírus da Hepatite B. A incidência desta neoplasia está a aumentar nos países Ocidentais, essencialmente relacionado com doenças hepáticas crónicas como a cirrose alcoólica e a infecção crónica pelo vírus da Hepatite C.

Epigenética refere-se a alterações na expressão génica reversíveis e hereditárias que são reguladas por mecanismos como a Hipermetilação das ilhas CpG, a Desacetilação das Histonas e RNAs de interferência. Nos últimos anos, as alterações epigenéticas têm sido associadas ao desenvolvimento do Carcinoma Hepatocelular, nomeadamente através da inibição de genes supressores tumorais, da activação de oncogenes e da instabilidade cromossómica. Seguindo esta linha de pensamento, pensa-se que fármacos moduladores Epigenéticos possam ter utilidade no tratamento do Carcinoma Hepatocelular.

Os objetivos deste trabalho de investigação são encontrar alterações epigenéticas em genes supressores tumorais, estudar o efeito de fármacos reguladores da epigenética na viabilidade de linhas celulares de Carcinoma Hepatocelular e verificar a reversão das epimutações após o tratamento farmacológico.

Recorrendo à técnica de PCR específica para a metilação, conseguimos demonstrar a existência de alterações epigenéticas em alguns genes relacionados com a apoptose e com a regulação do ciclo celular (*DAPK*, *PTEN*, *p16*) em 3 linhas celulares de Carcinoma Hepatocelular. Além disso, provámos a eficácia e sinergismo da Decitabina (um fármaco hipometilante) e da Trichostatin A (um fármaco inibidor da

Desacetilase das Histonas) na redução da viabilidade celular em linhas de Carcinoma Hepatocelular através do exame colorimétrico Alamar Blue. Verificámos, também, a reversão das alterações epigenéticas após o tratamento farmacológico com o estudo dos genes seguindo um protocolo de PCR específico para a metilação.

Este estudo reforça a ideia da existência de alterações epigenéticas no Carcinoma Hepatocelular e demonstra que os fármacos moduladores da epigenética podem ter um papel na terapêutica do Carcinoma Hepatocelular.

PALAVRAS CHAVE

Carcinoma Hepatocelular, Epigenética, Metilação de ilhas CpG, Acetilação de Histonas, DNA Metiltransferase, Desacetilase das Histonas, Decitabina, Trichostatina, Silenciamento de Genes Supressores Tumorais.

ABSTRACT

Hepatocellular carcinoma (HCC) is the second most frequent cause of cancer related deaths, with the heaviest burden on Southeast Asian and African countries, due to high rates of chronic Hepatitis B Virus (HBV) infection. The incidence of this tumor on Occidental countries is rising, essentially related to chronic liver diseases as the alcoholic cirrhosis and the chronic Hepatitis C Virus infection.

Epigenetics refers to heritable and reversible alterations on gene expression by regulatory mechanisms such as CpG island methylation, Histone Deacetylation and non-coding RNAs interference. Lately, epigenetic modifications have been pointed as being involved in HCC development through Tumor Suppressor Gene silencing, oncogene activation and chromosomal instability. Following this idea, it is thought that Epigenetic modulating drugs may pose a therapeutic option for HCC.

With this investigation work, we aimed to find epigenetic alterations on Tumor Suppressor Genes on Hepatocellular Carcinoma cell lines, to study the effect of Epigenetic modulating drugs on cell viability and to verify the reversion of epimutations after drug treatment.

By using a methylation-specific PCR protocol, we were able to find epigenetic alterations on some cell cycle regulator genes and apoptosis related genes (*p16*, *DAPK* and *PTEN*) on three different HCC cell lines. Additionally, we proved the efficacy and synergism of Trichostatin (a histone deacetylase inhibitor drug) and Decitabine (a hypomethylating drug) on reducing cell viability on HCC cell lines evidenced by Alamar Blue reduction assay. We also observed the reversion of promoter gene methylation after drug treatment.

This study reinforces the theory that epigenetic modifications are involved in Hepatocarcinogenesis and shows that epigenetic modulating drugs may be useful on HCC treatment.

KEYWORDS

Hepatocellular Carcinoma, Epigenetics, CpG islands Methylation, Histone Acetylation, DNA Methyltransferase, Histone Deacetylase, Decitabine, Trichostatin, Tumor Suppressor Gene silencing.

ABBREVIATIONS LIST

DAPK – Death Associated Protein Kinase

DEC - Decitabine

DMEM – Dulbecco's Modified Eagle's Medium

DNMT – DNA Methyltransferase

FBS – Fetal Bovine Serum

FITC – Fluorescein Isothiocyanate

GSTP1 – Glutathione S-Transferase P 1

H - Histone

HAT – Histone Acetyl Transferase

HBV – Hepatitis B Virus

HCV – Hepatitis C Virus

HCC – Hepatocellular Carcinoma

HDAC - Histone Deacetylase

IC50 – Half-maximal Inhibitory Concentration

Met – Methylation

NAFLD – Non Alcoholoic Fatty Liver Disease

PBS – Phostate Buffer Solution

PI – Propidium Iodide

PTEN - Phosphatase and Tensin homologue deleted on chromosome 10

RASSF1 – Ras associated domain-containing protein 1

SFRP1 - Secreted frizzled-related protein 1

TF – Transcription Factor

TSA – Trichostatin A

TSG – Tumor Suppressor Gene

1. Introduction

Since the unraveling of the Human Genome Sequence a decade ago, there has been a huge effort on linking specific gene sequences to specific phenotypes. Even if that led us to many close links and associations between genotype and phenotype, there are still many pathologic phenotypes unexplained by a specific DNA sequence[1]. These pathologies fall in many different fields, like endocrine regulation, learning, memory, neurological abnormalities, autism, type 2 diabetes, autoimmunity and cancer. The key for many of these pathologies may be found on Epigenetics [2-6.]

Epigenetics refers to reversible and heritable changes in gene expression caused by regulatory mechanisms, rather than changes in DNA sequence. All living cells in a single body have essentially the same genetic information; gene expression is what makes them different in phenotype and function. DNA base pairs are arranged in their smaller hierarchical unit, the nucleossomes, that gather up to form the chromatids that may be in different functional status in relation to gene expression. Active areas of the genome are found in regions of euchromatin, loosely packed, and more or less accessible to regulatory factors. Inactive areas are found as more densely packed heterochromatin, either constitutional or facultative[1]. Chromatin functional status is essentially defined by three epigenetic processes: DNA methylation, post translational Histone modification and non-coding RNA regulation[7].

In general, DNA methylation is associated with gene transcription silencing. It is related to several processes, like X chromosome inactivation, genome imprinting and repetitive sequences silencing[8]. A family set of enzymes is known to promote DNA methylation: DNA Methyltranferases - DNMT1, DNMT2 and DNMT3A and 3B. DNMT 1 is generally considered a maintenance methyltransferase responsible for passing DNA methylation patterns during DNA replication [9]. DNMT 3A and DNMT

3B are considered *de novo* methyltranferases, responsible for changes in DNA methylation pattern[10]. DNMT 2 is more related to RNA methylation than to DNA methylation, and is thought to represent the evolutionary origin of DNMT 1, 3A and 3B. DNA methylation refers to the addition of a methyl group to the Cytosine base pair of DNA, turning it to methyl-Cytosine. Methylation can occur in the CpG islands of the promoter region of the gene and repress gene transcription, mainly by inhibiting the binding of transcription factors[8] (Figure 1.1A). Hypermethylation of gene promoter sequence leads to gene expression suppression, and is generally associated to other epigenetic phenomena, like Histone Deacethylation and/or Methylation[8]. DNA promoter sequence demethylation is caused by enzymes like DNA Demethylases and is related to gene transcription activation (Figure 1.1B), and can be reinforced by Histone Acethylation and other epigenetic processes[11]. Intragenic DNA methylation is not well understood at the moment, and is thought to be a complex process.[12]

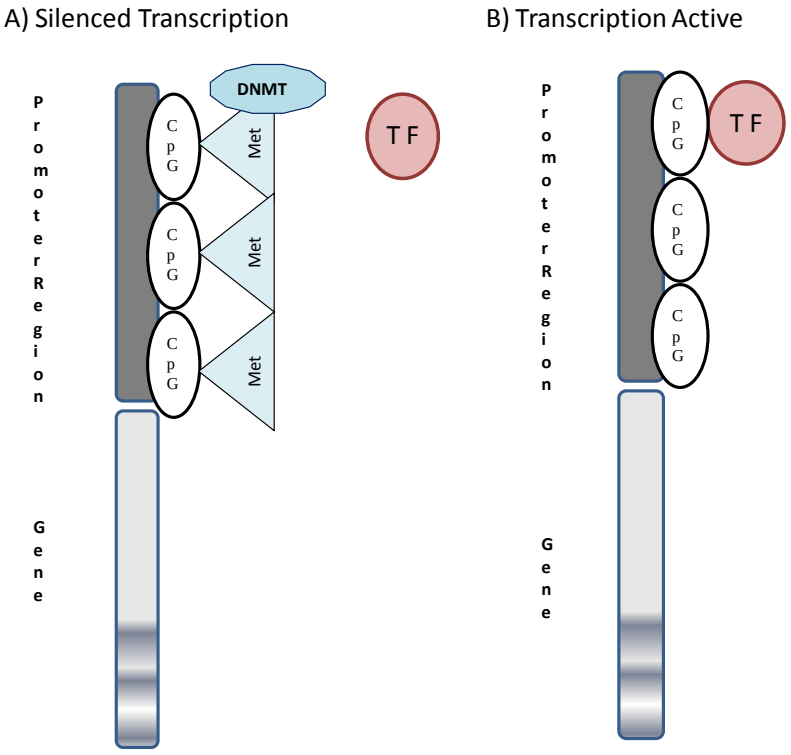

Figure 1.1 – Gene transcription regulation through DNA promoter sequence Methylation. A) Methylation (Met) of CpG islands (CpG) on the promoter region of the genes leads to gene transcription silencing by inhibiting the interaction of the Transciption Factors (TF) with the DNA sequence. B) When the promoter region of the gene is unmethylated, the Transcription Factors can interact with the DNA sequence and promote its transcription.

Histones are the proteins responsible for the basic morphology of DNA in nucleosomes. Being in such a short contact to DNA, they are also responsible for regulation on gene transcription by managing the condensation status of chromatin. Many of these processes are electrostatic in nature, and depend on post-translational small covalent modifications, methylation, acetylation, phosphorylation and others as

ubiquitination and sumoylation[13]. One of the most studied processes is Histone Acetylation and Histone Deacetylation, performed by the enzymes Histone Acetyl Transferase (HAT) and Histone Deacetylase (HDAC), respectively (Figure 1.2). Histone Acetylation leads to a conformational change in DNA, in which the electrostatic repulse "opens" the DNA sequence and allows the interaction with transcription factors (Figure 1.2A). Histone Deacetylases remove acetyl groups and are responsible for gene expression repression[14] (Figure 1.2B).

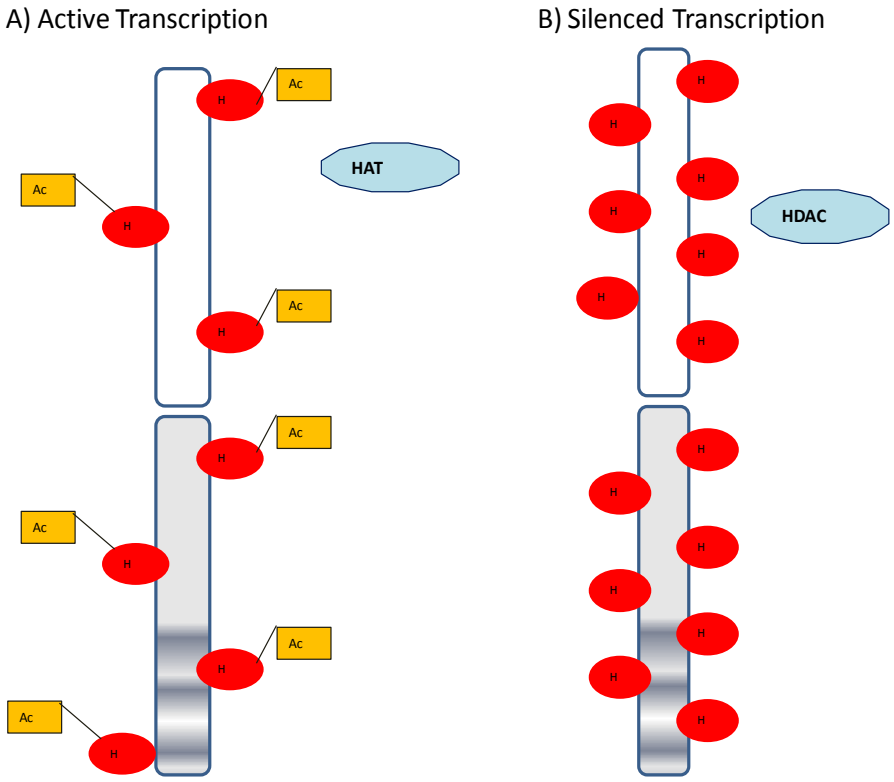

Figure 1.2 – Gene transcription regulation through Histone Acetylation. A) Histone Acetyl Transferase (HAT) are responsible for adding acetyl groups (Ac) to Histones (H), creating an electrical repulse that opens up the DNA conformation and allows gene transcription. B) Histone Deacetylase (HDAC) is responsible for removing the acetyl groups, closing the DNA sequence and leading to gene transcription silencing.

Finally, microRNAs (miRNA) are non-coding sequences transcribed in the nucleus and later exported to the cytoplasm[15]. They are involved in Epigenetics through their interaction with messenger RNAs (mRNAs), inhibiting their translation[15].

All these epigenetic processes are known to interact between each other in a complex balance and close crosstalk (Figure 1.3). It is well known, for example, the link between DNA Methylation and Histone Deacetylation, both concurring to gene transcription inhibition (Figure 1.3A). One cannot interfere in one regulatory system without making a change on the others[14].

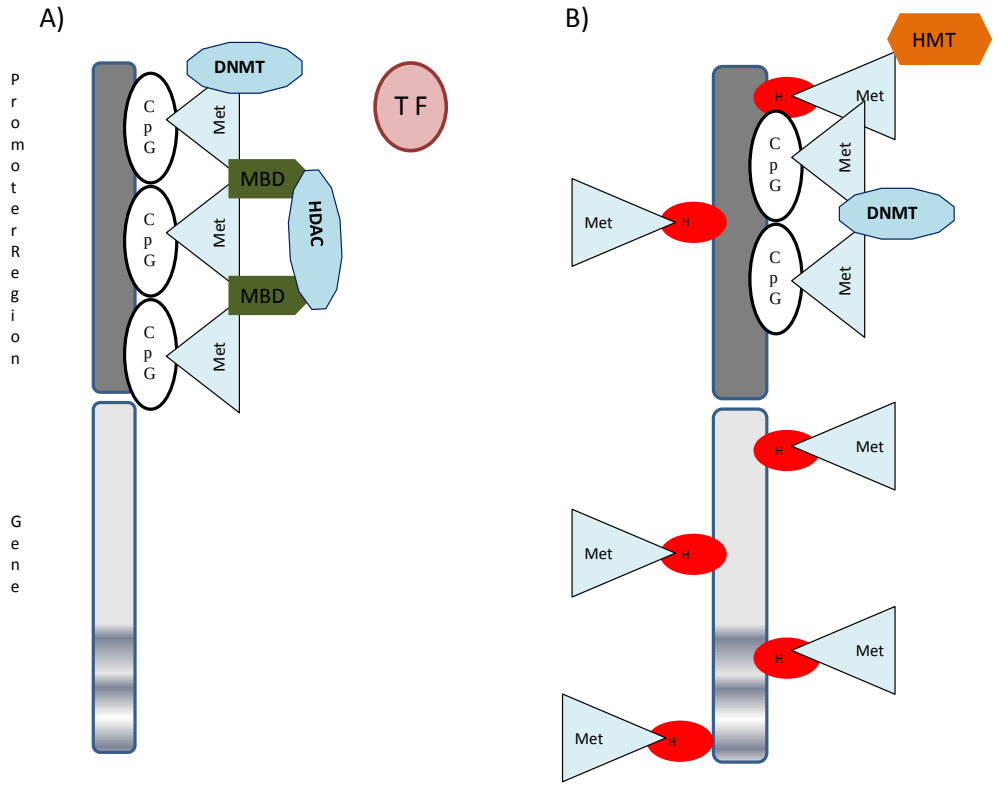

Figure 1.3 – Epigenetic regulatory mechanisms act in a close crosstalk that leads to a complex balance. A) CpG islands Methylation by DNMT leads to methyl-CpG

binding domain (MBD) proteins, which recruit Histone Deacetylase (HDAC) to induce transcriptional repression by deacetylation of histones. This inhibits binding of Transcription Factors (TF). B) Histone (H) methylation (Met) by Histone Methyl Transferase (HMT) leads to *de novo* DNA Methylation and subsequent gene transcription repression[14].

This close control of gene transcription played by Epigenetics makes it responsible for the plasticity of cells. However, such a complex balance may be disrupted, which leads to pathology, namely cancer. Many epigenetic modifications leading to cell proliferation have been discovered. Since they are functionally related to genetic mutations, they became known as epimutations [14]. Some examples are: DNA global hypomethylation[16], which leads to chromosomal instability, protooncogene activation and activation of retrotransposons; promoter tumor suppressor gene hypermethylation[17] and Histone Deacetylase increased activity[15], leading to tumor suppressor gene silencing by suppression of transcription.

This link between Epigenetics and pathology seems to be a disruption on the complex balance in which the epigenetic modulating enzymes act[14]. The fact that Epigenetics is a reversible process makes it a target for drug therapy on cancer[14]. DNMTs are the target for inhibition by drugs like 5-Azacytidine and 2'-Deoxy-5-azacytidine (Decitabine). By inhibiting DNMTs, these drugs lead to DNA hypomethylation and possible activation of tumor suppressor genes. Both drugs are already approved for clinical use in Myelodysplastic Syndrome. Several groups of Histone Deacetylase inhitors have been discovered over the past years [14] (hydroxamic acids – Trichostatin (TSA), Panobinostat or Vorinostat (…); Cyclic tetapeptides

(Istodax); Short chain fatty acids (Valproic acid); Benzamides (Entinostat, Mocetinostat); and Synthetic benzamides (N-acetyldinaline). By inhibiting Histone Deacetylase, they are thought to keep Histone acetylated, hence gene transcription active. Some of them are in running clinical trials for several oncologic conditions [14], and, for example, Vorinostat is already approved for Cutaneous T cell Lymphoma. However, clinical evidence for the benefit of these drugs wasn't based in concrete pharmacodynamics studies. In fact, it is known that DNMT inhibitors are not substract specific [18], and that HDAC enzymes are not Histone specific [19]. Therefore, the rationale for the use of these drugs still needs to be proven.

Hepatocellular carcinoma (HCC) is the second most frequent cause of cancer related deaths, with the heaviest burden on Southeast Asian and African countries, due to high rates of chronic Hepatitis B Virus (HBV) infection and Aflatoxin 1 ingestion [20]. In Occidental countries, the incidence of HCC is rising due to chronic liver disease, such as Hepatitis C Virus infection, Non Alcoholic Fatty Liver Disease (NAFLD) and alcohol abuse. The incidence rate closely equals the mortality rate [21].

High risk patients should be screened every 6 to 12 months through serum Alfa-Fetoprotein and liver ultrasound [21]. This surveillance has led to a modest increase in survival rate in the United States [21]. Diagnosis is usually made in a high risk patient through imaging studies, like Computed Tomography, that show specific features related to HCC blood supply.

Many treatment options are available, depending on staging: surgical resection, liver transplant, Transarterial embolization (bland particle and chemoembolization), Ethanol Injection, Cryoablation, Radiofrequency Ablation and Chemoterapy [21]. Unfortunately, many patients are diagnosed in a late stage, when chemotherapy is the

only option and the survival expectancy is very low – 4 months [21]. To date, chemotherapy for HCC is limited to one drug – Sorafenib, the only drug able to show a survival advantage of 3 months for the treated patients [22-24]. Sorafenib has also been used in an adjuvant setting, with good results [25]. In a neoadjuvant setting, Sorafenib is not so promising, since its antiangiogenic effects may impair further liver-directed therapies [21] .

Besides the connection between some risk factors and HCC, much is still left to learn about Hepatocarcinogenesis. It is generally accepted that the progression from a normal cell to a neoplastic cell involves the loss of tumor suppressor genes and the activation of protooncogenes. The original idea was that genetic mutations were the cause for this transformation. However, in HCC, studies show that mutation of some tumor suppressor genes, such as p53, B-catenin and Axin are found only in 20% to 30% of tumor samples, while abnormal methylation of tumour suppressor genes, such as *p16INK4a*, *E-cadherin*, *SFRP1*, *GSTP1* ad *RASSF1A* is observed in the promoter regions of more patients' samples [26]. Universal Hypomethylation of HCC genome has also been implied in Hepatocarcinogenesis, in animal models and in tumour samples [27-28]. Universal Hypomethylation is thought to be related to chromosomal instability, transposon elements activation[26] and activation of protooncogenes, like c-myc[29] . Hypermethylation of promoter sequence of tumor suppressor genes has also been connected to Hepatocarcinogenesis: *p16INK4a* was methylated in 48% of 26 tumor samples[30] and another study related this finding to HBV infection[31]; *RASSF1A*, a gene related to DNA repair was found to be hypermethylated in 85% of 83 tumor samples and was related to aflatoxin B1 exposure[32]. Differences have been found in DNMT 3B expression between HCC samples, cirrhotic liver samples and normal liver tissue samples, suggesting tumor suppressor hypermethylation as an early event in

hepatocarcinogenesis[33]. Research data also support the involvement of Histone modifications on Hepatocarcinogenesis: HDAC1 and SIRT1 (a member of the HDAC3 family) have high expression levels on invasive HCC samples[34-35]. Inflamation, as well as HBX, a gene incorporated by Hepatitis B Virus on the DNA of host cell, have also been related to alterations on the Epigenome[36]. All these data reinforce the interest on Epigenetics as a new target on Hepatocellular carcinoma.

With this study, we aimed to search for epigenetic mutations on tumor suppressor genes on Hepatocellular Carcinoma cell lines, to study epigenetic modulating drugs effect on cell viability and cell death, and finally, to verify the reversion of the epimutations after drug treatment.

2 - Material and Methods

2.1 - Cell lines

In our studies we used 3 HCC cell lines, the HUH-7, HepG2 and Hep3B cells, obtained from different HCC samples with different etiologies and with different p53 levels.

HUH-7 cell line is an immortal well differentiated epithelial-like tumorigenic cell line originally taken from a liver tumor (HCC) of a 57 years old Japanese male in 1982 and established by Nakabayshi, H. and Sato, J. as a model of HCC with p53 overexpression. This cell line was offered by Professora Doutora Maria Conceição Pedroso Lima (Center for Neuroscience and Cell biology).

HepG-2 is a cell line that was first obtained from the liver tissue of a fifteen years old Caucasian American male diagnosed with Hepatocellular Carcinoma that presents normal expression of p53. This cell line was offered by Professora Doutora Filomena Botelho, from Biophysics/Biomathematics of the Faculty of Medicine, University of Coimbra.

Hep3-B has been isolated from a liver tumor biopsy of an 8 years old boy in 1976, contains an integrated hepatitis B virus genome and does not express p53 due to partial deletion in the p53 gene locus. This cell line was offered by Professora Doutora Filomena Botelho, from Biophysics/Biomathematics of the Faculty of Medicine, University of Coimbra.

Cell lines were maintained in DMEM medium (Gibco – Life Technologies) supplemented with 10% heat inactivated Fetal Bovine Serum (FBS) (Gibco – Life Technologies), L-glutamine 2mM, $NaHCO_3$, penicilin 100U/mL and streptomycin

100µg/mL at 37°C in a humidified incubator containing 5% CO_2. For the experiments, cells were seeded at a density of 50000 cells per cm2.

2.2 - HCC cell lines gene methylation patterns.

DNA was extracted from the 3 cell lines using the Illustra tissue and cells genomicPrep Midi Flow Kit® from GE Healthcare as indicated by the manufacturer. DNA methylation of *p15*, *p16*, *p21*, *PTEN* and *DAPK* genes was determined by chemical treatment with sodium bisulfite using the EpiTect Bisulfite kit® from Qiagen and subsequent PCR using specific primers for methylated and unmethylated DNA promoter sequence as previously described by other authors[45] (see next section for primers and PCR conditions). All PCRs were performed with positive and negative methylation DNA control. PCR products were run on a 10% agarose gel and visualized by staining with ethidium bromide.

2.3 - **PCR: conditions, primers and annealing temperature**

The PCR conditions are shown in Table 2.1:

Table 2.1 – PCR reaction conditions

Reagent	**Volume / well**
PCR Buffer	2 µL
Q solution	4 µL
Nucleotides	2 µL
$MgCl_2$	4,20 µL

Forward Primer	0,8 µL
Reverse Primer	0,8 µL
Taq Polymerase	0,5 µL
H_2O	3,7 µL
Cell's DNA	2 µL
Total	20 µL

The PCR primers and Temperatures used in Methylation Specific PCR were:

p15-UF TGTGATGTGTTTGTATTTTGTGGTT (25 bp)

p15-UR CCATACAATAACCAAACAACCAA (23 bp)

Annealing temperature: 60°C

p15-MF GCGTTCGTATTTTGCGGTT (19 bp)

p15-MR CGTACAATAACCGAACGACCGA (22 bp)

Annealing temperature: 60°C

p16-UF TTATTAGAGGGTGGGGTGGATTGT (24bp)

p16-UR CCACCTAAATCAACCTCCAACCA (23 bp)

Annealing temperature: 60°C

p16-MF TTATTAGAGGGTGGGGCGGATCGC(24bp)

p16-MR CCACCTAAATCGACCTCCGACCG (23bp)

Annealing temperature: 65°C

DAPK-UF GGAGGATAGTTGGATTGAGTTAATGTT (27bp)

DAPK-UR CAAATCCCTCCCAAACACCAA (23 bp)

Annealing temperature: 60°C

DAPK-MF GGATAGTCGGATCGAGTTAACGTC (24 bp)

DAPK-MR CCCTCCCAAACGCCGA (16bp)

Annealing temperature: 60°C

PTEN(UF) TATTAGTTTGGGGATTTTTTTTTTGT (27 bp)

PTEN(UR) CCCAACCCTTCCTACACCACA (23bp)

Annealing temperature: 60°C

PTEN(MF) GTTTGGGGATTTTTTTTTCGC (21 bp)

PTEN(MR) AACCCTTCCTACGCCGCG (19 bp)

Annealing temperature: 60°C

p21-MF TACGCGAGGTTTCGGGATCG (20 bp)

p21-MR AAAACGACCCGCGCTCG (17 bp)

Annealing temperature: 61°C

p21-UF TATGTGAGGTTTTGGGATTGG (22 bp)

p21-UR AAAAACAACCCACACTCAACC (21 bp)

Annealing temperature: 61°C

All PCR primers were acquired from Sigma Aldrich, St. Louis, MO, USA.

2.4 – Epigenetic modulating drugs effect on cell viability.

To determine the drug dose dependent changes in cell viability, cells were cultured in the absence (control) and presence of 5-aza-2dC (Decitabine) (concentration range: 1µM to 50 µM) (Sigma Aldrich, St. Louis, MO, USA) and/or Trichostatin (concentration range: 10nM to 500 nM) (Sigma Aldrich, St. Louis, MO, USA), for up to 72h. No further addition of drug was made after the first dose.

To check for possible synergistic effect, both drugs were used simultaneously (TSA – 100nM + DEC - 1 µM) and compared to their use in monotherapy.

To analyze the possible effect of the drug administration schedule, we have done experiments using the drugs administered simultaneously and separate by four hours - each one of the drugs was added first and the second one only 4 hours later, and vice-versa (TSA – 100nM; DEC - 1 µM). The antiproliferative effect was assessed by Alamar Blue assay (Resazurine, Sigma Aldrich, St. Louis, MO, USA) each 24h, during 72h. In this assay, cells treated with the different conditions are incubated with Alamar Blue (Resazurine at 0,1 mg/mL in PBS) 10% (v/v) in DMEM, for 2 hours at 37°C [46]. After that period, 200µL of supernatant are collected from each well and transferred to 96 well-plates. The absorbance at 570nm and 600nm is measured using a Mediators PhL luminometer (Mediators Diagnostika, Vienna, Austria) and cell viability is calculated as a percentage of control according to the formula:

$$\frac{[(A_{570} - A_{600})sample] - [(A_{570} - A_{600})blank]}{[(A_{570} - A_{600})control] - [(A_{570} - A_{600})blank]} \times 100$$

The IC50 value (drug concentration to attain 50% inhibition of cell viability) was calculated from three independent experiments using GraphPad Prism 4.00.

2.5 - Cell death evaluation by Morphological analysis

After incubation for 48h with both drugs alone and in association (DEC 1 µM, TSA 200 nM, DEC 1 µM + TSA 100 nM), HUH-7 cells were trypsined, centrifuged at 300xg for 5min and ressuspended in serum in order to obtain a density of 50000cells/mL. Then, HUH-7 cells were stained with May-Grünwald solution (0.3% v/v in methanol) (Sigma, St. Louis, MO, USA) diluted in 1:1 ratio with distilled water followed by staining with Giemsa solution (0.75% p/v in glycerol/methanol 1:1) (Sigma, St. Louis, MO, USA) diluted 8x in distilled water for 20 min. After rinsed with distilled water, smears were left to dry at room temperature. The cells' morphology was analyzed by light microscopy using a Leitz Dialux 20 microscope associated with a Moticam 2300 digital camera.

2.6 - Cell death analysis by flow cytometry

HUH-7 cells were cultured in the absence or in the presence of the drugs (DEC - 1 µM; TSA – 200nM; TSA+DEC – 100nM + 1µM). At 48 hours, they were trypsinized, centrifuged at 300xg for 5min and incubated for 10 min at 4ºC with 440µL annexin buffer containing 5µL FITC-labelled Annexin V (Kit from Immunotech SA, Marseille, France) and 2µL Propidium Iodide (PI)[47]. Cells were then washed twice with PBS, ressuspended in the same buffer and analyzed in a FACScalibur cytometer (BD Biosciences, Heildelberg, Germany) equipped with an argon ion laser emitting at 488nm. The fluorescence of AV-FITC and PI was evaluated at 525 and 610nm, respectively.

Annexin V binds with high affinity to phospholipids negatively charged including phosphatidylserine which is exposed in the outer leaflet of the plasma

membrane during apoptotic process. PI is a non-specific DNA marker which is internalized by cells that lost membrane integrity. With this technique, it is possible to distinguish non-apoptotic live cells (AV-FITC and PI negative), early apoptotic cells (AV-FITC positive and PI negative), late apoptotic (positive for FITC-AV and PI) and necrotic cells (positive for PI and AV-FITC negative).

The results were expressed as percentage of live, early apoptotic, late apoptotic/necrotic and necrotic cells according to their rate of fluorescence on both light wave length.

2.7 – Gene Methylation pattern reversion

The cells were cultured in the absence (control) and presence of DEC (concentration 1 µM), TSA (concentration 200 nM) and DEC plus TSA (concentration 1 µM plus 100 nM, respectively).

Then, DNA was extracted from the experiment using the Illustra tissue and cells genomicPrep Midi Flow Kit® from GE Healthcare as indicated by the manufacturer. In treated cells, *p16* DNA methylation was determined by chemical treatment with sodium bisulfite using the EpiTect Bisulfite kit® from Qiagen and subsequent PCR using specific primers for methylated and unmethylated DNA promoter sequence was performed[45] (see section 2.3). PCRs were performed with positive and negative methylation DNA control. PCR products were run on a 10% agarose gel and visualized by staining with ethidium bromide as previously referred

2.8 - Data Analysis

Statistical analyses were performed using GraphPad Prism software, version 4.0 (GraphPad Prism software, Inc., San Diego, CA).

Data are expressed as mean ± SD obtained from independent determinations, each one performed in duplicate or triplicate. Differences between data sets were determined by ANOVA test. A p value <0.05 was considered as statistically significant.

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3 - Results

3.1 Evaluation of gene Methylation pattern on different HCC cell lines

In order to study the role of Epigenetics on Hepatocarcinogenesis, we studied gene methylation patterns of some Tumor Suppressor Genes (TSG) on 3 HCC cell lines, HUH-7, HepG2 and Hep3B. Methylation of gene promoter is one of the most studied Epigenetic processes and leads to gene transcription inactivation.

Our results represented in Figure 3.1 show different patterns of methylation between the different HCC cell lines used. While HepG2 and Hep3B cell lines showed methylation of *PTEN*, *DAPK* and *p16*, HUH-7 showed methylation of *DAPK* and *p16*. In all cell lines, *p21* and *p15* were unmethylated.

Other genes were studied, like *p53*, *GSTP1* and *RASSF1*, but no results were found, probably related to the PCR protocol (DNA probes).

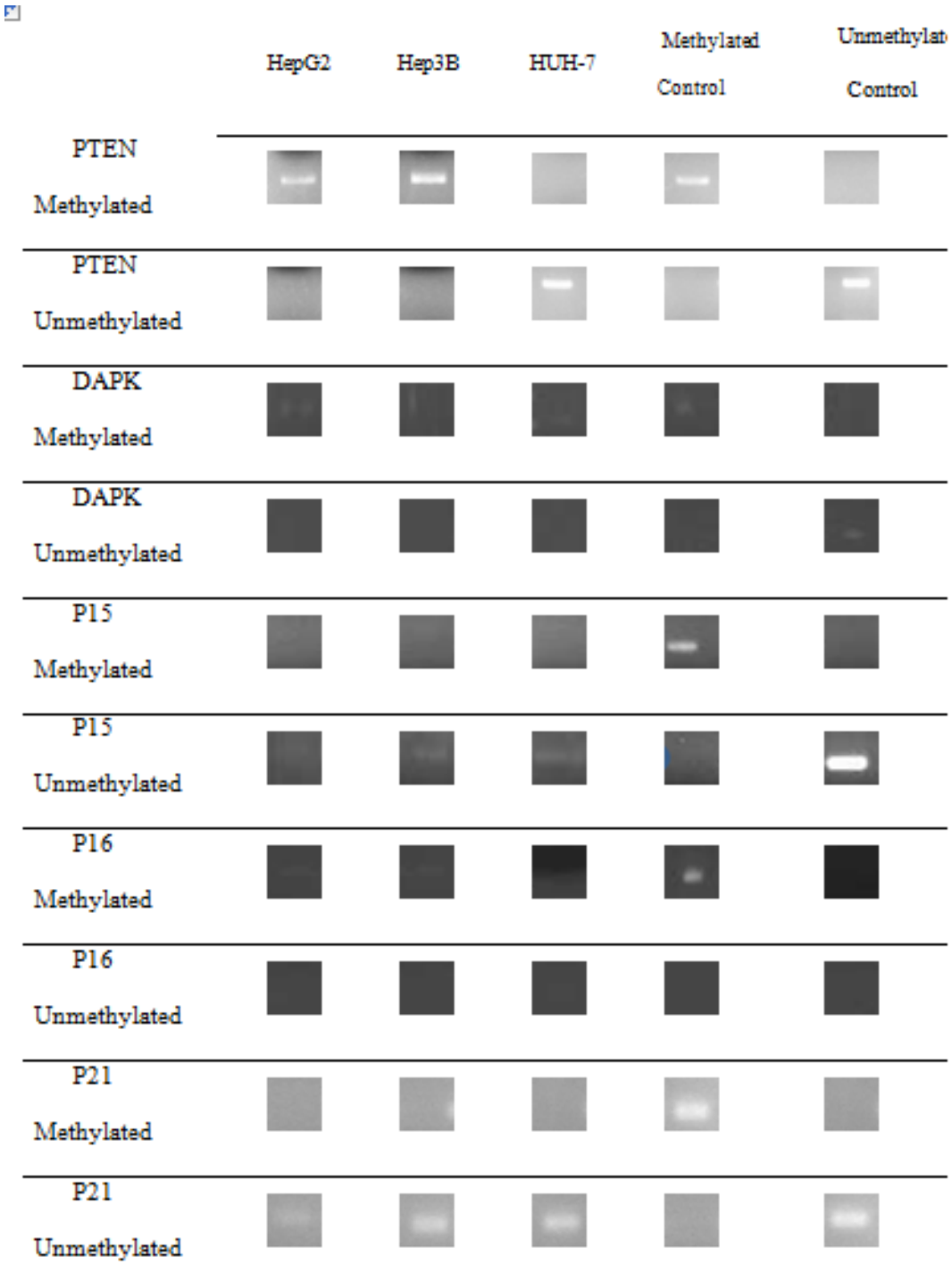
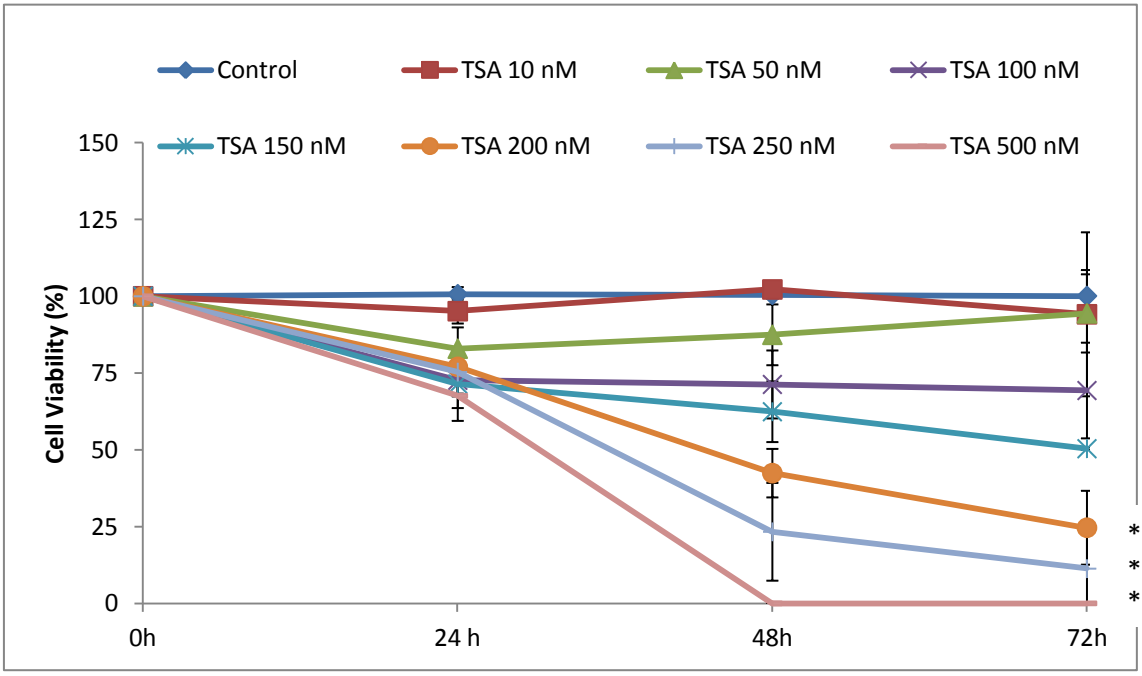


Figure 3.1 – Gene Methylation pattern in the HCC cell lines. HepG2 and Hep3B cells showed methylation of *PTEN*, *DAPK* and *p16* gene promoter sequence. HUH-7 showed methylation of *DAPK* and *p16* gene promoter sequence.

3.2 – Epigenetic modulating drugs effect on cell viability.

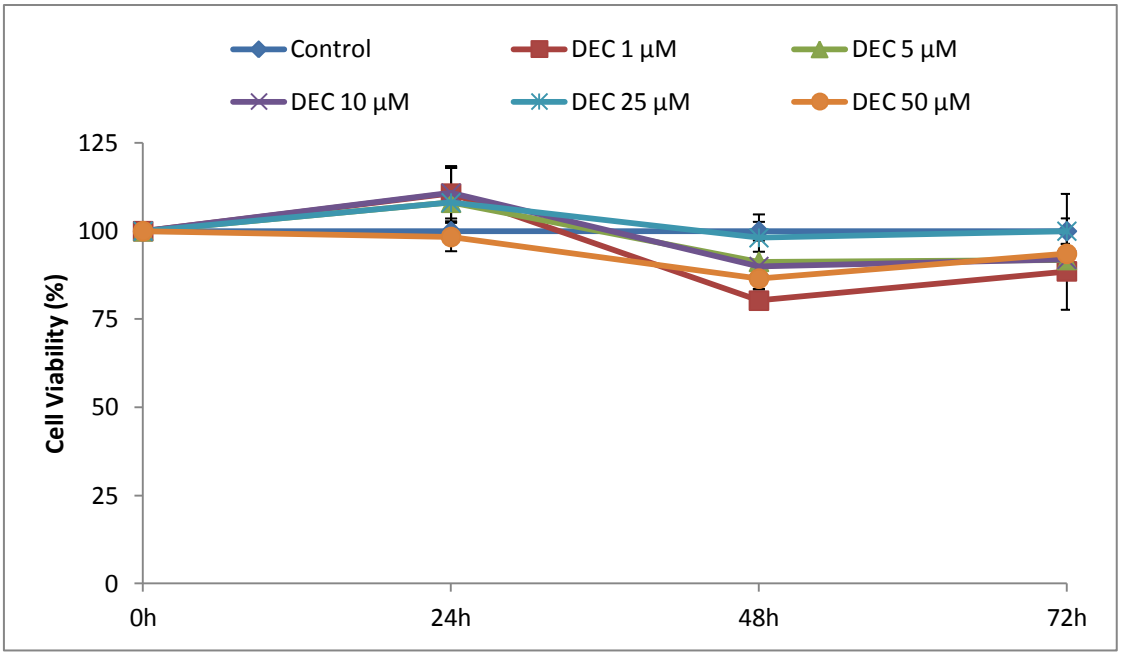
Since epigenetic modifications have been shown to play a role on carcinogenesis, it is believed that drugs acting in reversible processes like DNA Methylation and Histone Deacetylation can restore normal epigenetic regulation, namely TSG re-expression leading to cancer cells' death. To study this hypothesis, we used two different epidrugs. Trichostatin A (TSA), a Histone Deacetylase inhibitor, expected to inhibit Histone deacetylation and 2'Deoxy – 5'azacytidin (Decitabine – DEC) is a DNA Methyltransferase inhibitor, expected to lead to DNA hypomethylation and reactivation of TSG. For this purpose, both drugs were used alone and in association in different concentration ranges and administration schedules, in HUH-7 cell line.

Our results show that Trichostatin (Graph 3.1) has the potential to reduce cell viability in a time and dose dependent manner for concentrations above 100 nM on HUH-7 cell line. The decrease in cell viability happens to all concentrations at 24 hours of incubation, but is more pronounced after 48 hours ($p<0.05$), where we found the IC 50 value of approximately 200nM. After the 48 hours period, there is still a decrease in cell viability for the 100nM, 150nM, 200nM and 250 nM concentrations; there were no viable cells after 48 hours for the 500nM concentration.



Graph 3.1 – Dose and time response in HUH-7 cell line treated with Trichostatin (TSA). Cells were cultured in the absence (Control) and presence of TSA in the concentrations represented in the Graph as described in Material and Methods during 0, 24, 48 and 72 hours. The IC 50 value at 48 hours was approximately 200 nM. The results are expressed in percentage (%) and represented the mean ±SD of 3 independent experiments. (** P<0.05)

When HUH-7 cells were treated with Decitabine alone (Graph 3.2), at 24 hours of experiment, all concentrations, except 50 µM, showed an increase in cell viability. Only after 48 hours a slight decrease in cell viability was observed for all drug concentrations, where the lowest concentration used (1 µM) induces the higher decrease in cell viability, however this decrease is only around 20%. After 72 hours, we observe a reversion of this effect, as all concentrations used presented an increase in cell viability (above 85%), but still less than control.

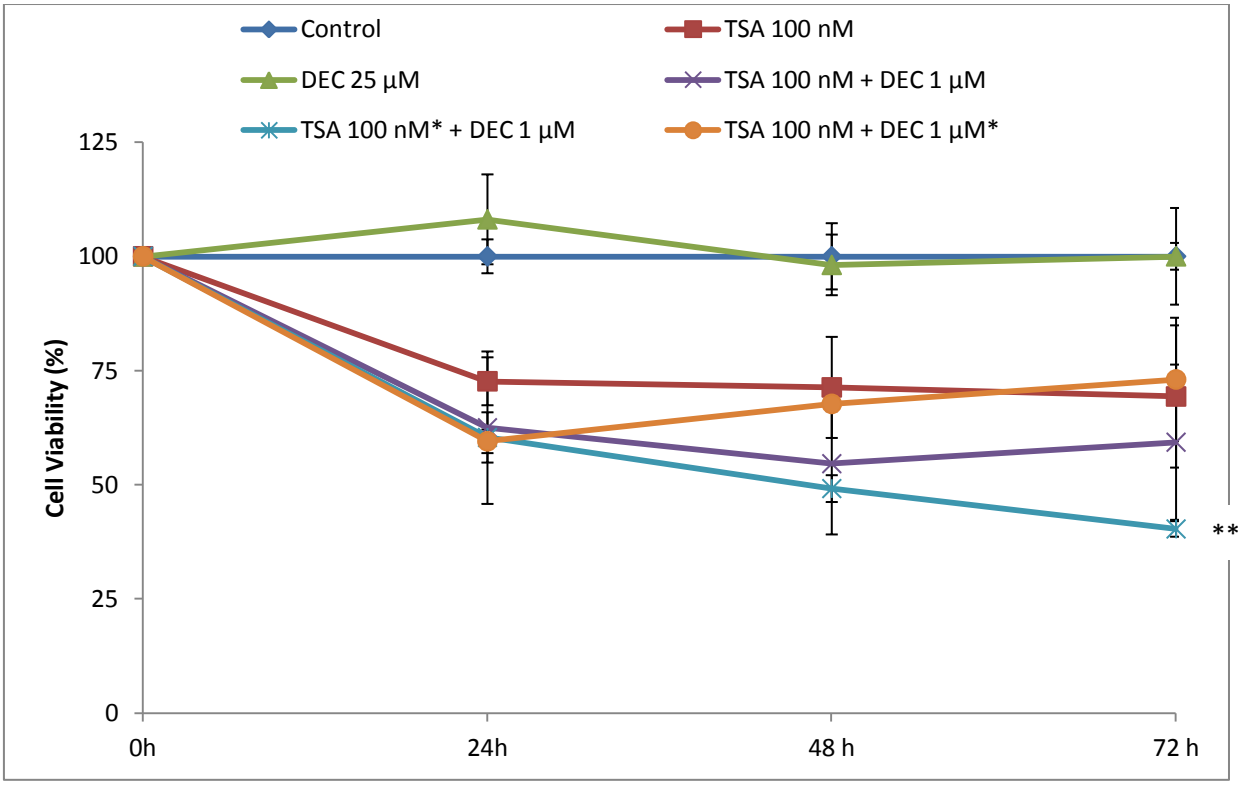


Graph 3.2 – Dose and time response in HUH-7 cell line treated with Decitabine (DEC). Cells were cultured in the absence (Control) and presence of Decitabine in the concentrations represented in the Graph as described in Material and Methods during 0, 24, 48 and 72 hours. The results are expressed in percentage (%) and represented the mean ±SD of 3 independent experiments

To study the possibility of a synergistic effect between the two epidrugs used, the cells were treated with the combination of Trichostatin A and Decitabine in concentration below the IC50 obtained with TSA in monotherapy (100 nM for TSA and 1 µM for Decitabine) as represented in Graph 3.3.

As we can observe in Graph 3.3 the simultaneous association of TSA (100nM) and DEC (1µM) produced a slight decrease in cell viability when compared to Trichostatin A (100nM) alone.

Then, we studied the possible effect of adding the drugs, with the same concentration, in a sequential way, being the administration of each drug separated by four hours. Our results show that the sequential incubation of Trichostatin A before Decitabine failed to prove to be more effective than either each agent alone. However, after 72 hours of incubation, a very significant reduction of cell viability was observed when DAC was administered before TSA ($p<0.05$). In this condition we observe a reduction of cell viability about 60% (Graph 3.3)

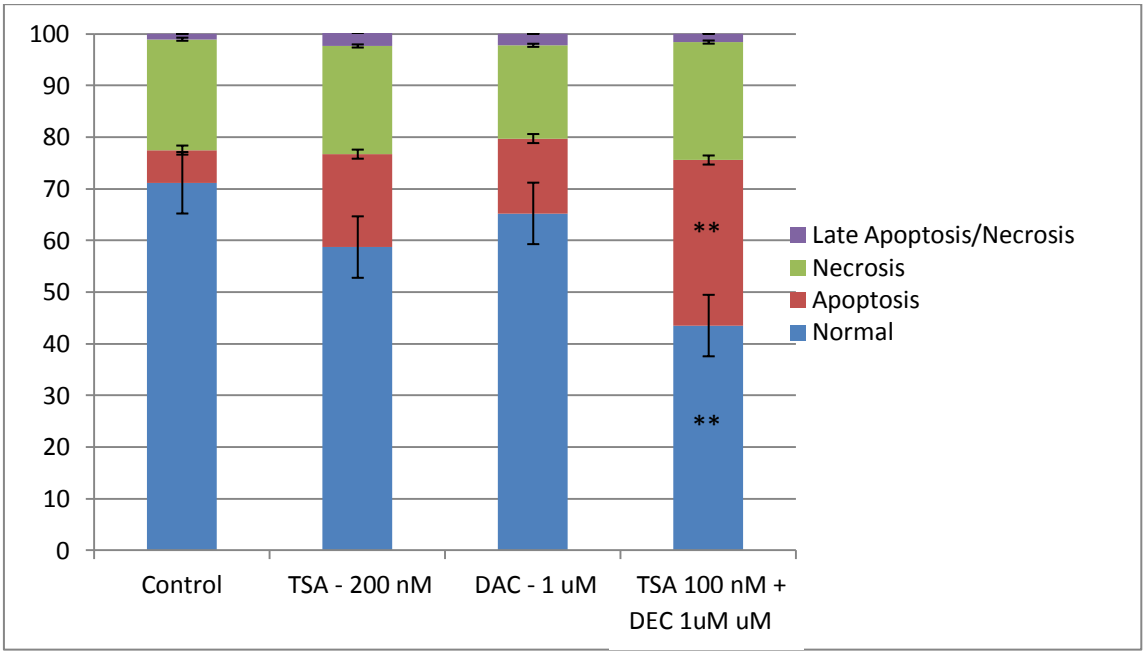


Graph 3.3 - Dose and time response curve in HUH-7 cell line treated with the association of TSA and DEC. Cells are cultured in absence (control) and presence of TSA plus DEC in the concentrations presented in the Graph, simultaneously and with an interval of 4 hours (*drug added for hours later) as described in material and methods, during 0, 24, 48 and 72 hours. The results are expressed in percentage (%) and represented the mean ±SD of 3 independent experiments. (**$p<0.05$)

3.3 - Study of cell death by flow cytometry

To study cell death mechanisms in HUH-7 cells treated with epigenetic modulating drugs, we used Flow Cytometry, after labeling the cells with Annexin V and Propidium Iodide.

Our results (Graph 3.4) show that TSA (200nM) and DEC (1µM), in monotherapy, induce a decrease in cell viability about 42% and 35%, respectively, which is accompanied by an increase in cell death mainly by apoptosis and necrosis, more evident in cells treated with TSA (Apoptosis about 19% and Necrosis 21%). When the cells are treated with TSA (100nM) in combination with DEC (1µM) a synergistic effect is obtained, as we observe a significant increase in percentage of death cells (57%) when compared with cells treated with the drugs in monotherapy (Apoptosis about 34,2% and Necrosis 22,8%)



Graph 3.4 - Cell death analysis by Flow Cytometry using annexin V and propidium iodide labeling. HUH-7 cells were incubated in the absence (control) and in

the presence of 200nM TSA and 1 µM DEC in monotherapy and in association [TSA (100nM) + DEC (1 µM). (**p<0.05)

3.4 - Cell death evaluation by Morphological analysis

In order to confirm the mechanisms of cell death obtained in flow cytometry studies, HUH-7 cells' morphology was analyzed through optic microscopy (Figure 3.2) without drug treatment (Ctl) (Figure 3.2-A) and 48 hours after drug treatment with DEC 1 µM (Figure 3.2-B) and TSA 200nM (Figure 3.2-C) alone and in combination (DEC 1 µM plus TSA 100nM) (Figure 3.2-D). In the cells treated with the drugs alone (Figure 3.2-B and C) and in combination, (Figure 3.2-D) we can observe features of apoptosis, such as nuclear fragmentation, blebbing and apoptotic bodies formation. Some necrotic cells with total membrane disruption were also found, mainly in the cells treated with Trichostatin.

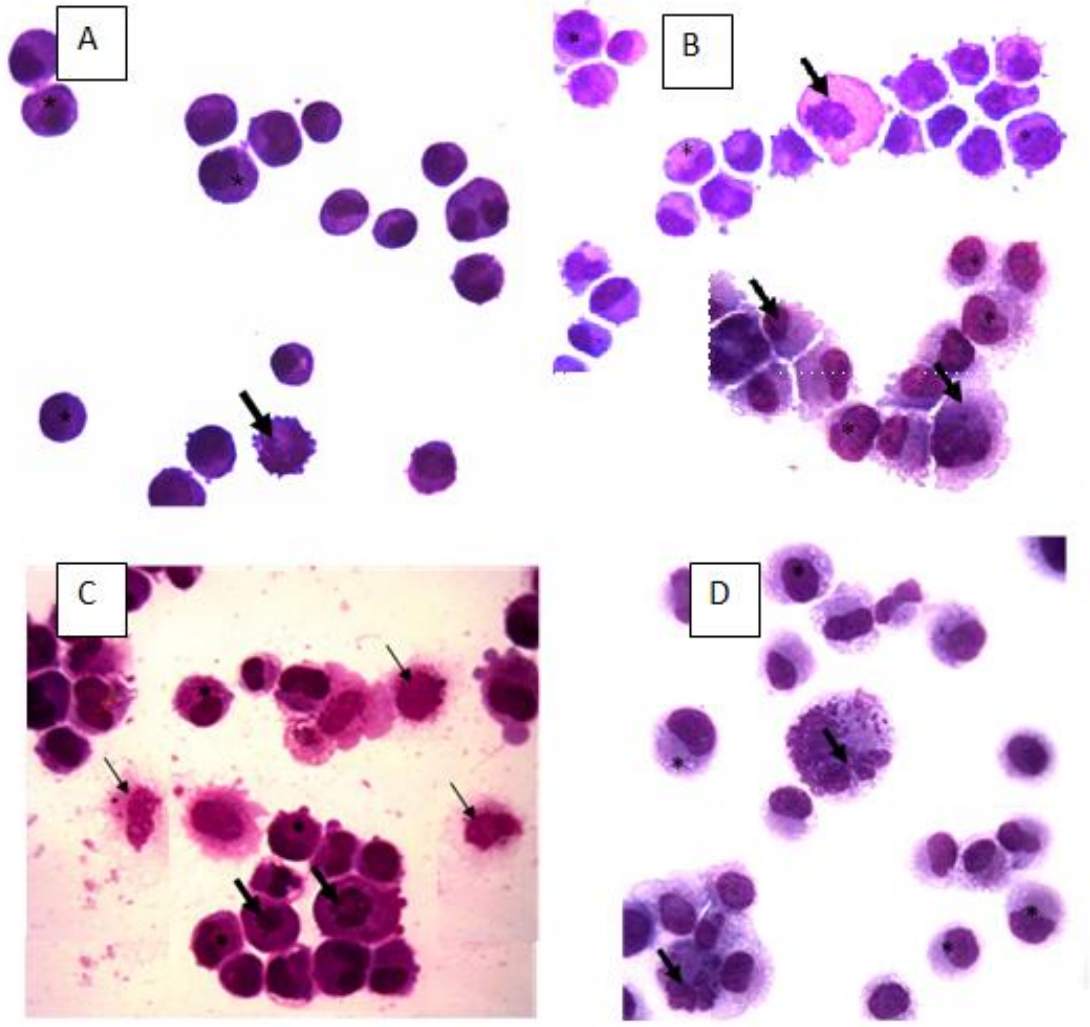


Fig. 3.2 - Morphological analysis of HUH-7 cells by optical microscopy. Cells were incubated in absence (Control, **A**) and in presence of Decitabin 1 µM (**B**), Trichostatin 200 nM (**C**) and in combination with both drugs TSA 100nM + DEC 1 µM (**D**). After cells were stained with May-Grünwald-Giemsa as refereed in Material and Methods. Apoptotic cells (dark arrow) were more frequent than in the control. Light arrow – Necrotic cells. * - Normal cells. Amplification: 400x.

3.5 – Gene Methylation pattern reversion

To study the effect of Epigenetic modulating drugs in the reversion of gene hypermethylation, cells were incubated with TSA 200nM, DEC 1 µM and TSA 100nM plus DEC 1 µM and later their DNA was extracted. The *p16* gene previously detected in control as a methylated gene was studied by a Methylation specific PCR (Figure 3.3) using methylated (Figure 3.3-A) and unmethylated primers (Figure 3.3-B) After treatment with the studied epidrugs we found that Decitabine (DEC) 1 µM was able to decrease *p16* gene methylation. shown by a slight decrease in the electrophoretic band (Figure 3.3-A). Thrichostatin 200 nM (TSA) alone and Thrichostatin 100 nM plus Decitabin 1 uM (T+D) didn't show a decrease in the gene methylation profile, when compared to Control (CTL) and with the Methylated Control (M-C). When we analyze the unmethylated gene, we found that the TSA 100nM in association with DEC 1 µM (T+D) were able to create a light band (Figure 3.3-B). As the control (CTL) didn't show any band for the unmethylated gene, this light band in cells treated with T+D may be related with the capacity of this combination of drugs to produce some hypomethylation in the HUH-7 cell line.

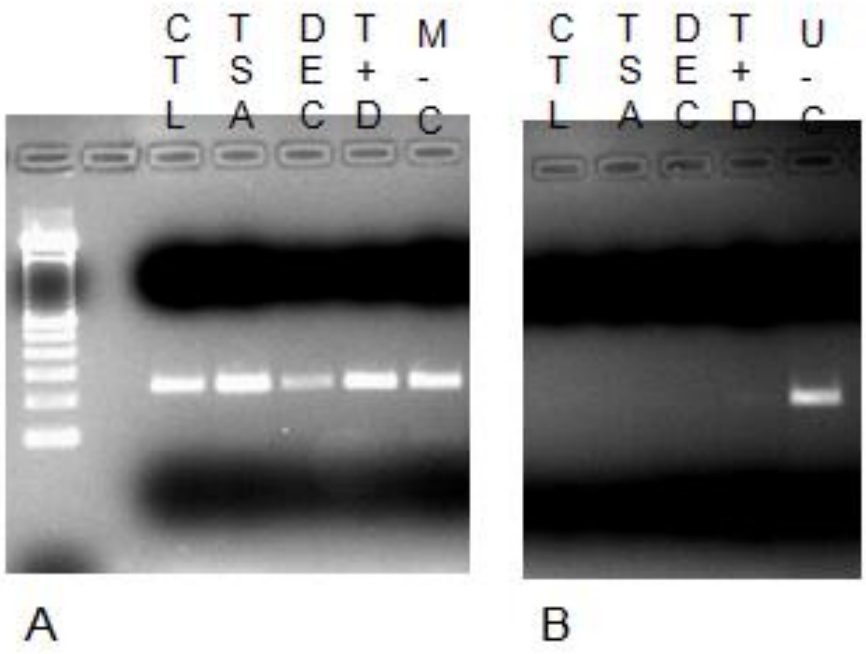


Figure 3.3 - p16 gene methylation pattern analysis performed in HUH-7 cells treated with Thrichostatin and Decitabine. Cells were treat in absence (CTL) and presence of Decitabine 1 uM (DEC), Thrichostatin 200 nM (TSA) and Thrichostatin 100 nM in combination with Decitabine 1 uM (T+D). The methylation status was performed by MS-PCR as described in Material and Methods using **methylated (A) and unmethylated (B) primers and** compared to Control (CTL) and to Methylated and Un-Methylated Controls (M-C and U-C, respectively).

4 – Discussion and Conclusions

In recent years, the interest on Epigenetics has been renewed. Epigenetics is not related only with embryogenesis, development and genetic imprinting, but has gained a new interest, especially in the always growing field of Oncology.

One of the essential steps for interference of Epigenetics in Oncology is tumor suppressor gene (TSG) hypermethylation. This process is responsible for gene transcription silencing and so, for the loss of function of these TSG, which are essential in the control of cell proliferation and death. Several genes have been implicated[7, 15] as *p16*, *Death Associated Protein Kinase* (*DAPK*) and *PTEN* (*Phosphatase and Tensin homologue deleted on chromosome 10*).

p16 is a cdk4 and cdk6 inhibitor responsible for cell cycle arrest in G1 phase. This TSG inhibits the CyclinD1/cdk4 complex, therefore inhibiting pRB phosphorylation [37]. Many authors[30, 31] have implicated *p16* epigenetic regulation in Hepatocarcinogenesis. *Death Associated Protein Kinase (DAPK)* codifies a protein with the same name which is a calmodulin regulated and cytoskeleton-associated serine/threonine kinase[38]. *DAPK* is thought to be a TSG for its potential to promote apoptosis through p53 pathway [39] and for its ability to inhibit E2F and c-MYC dependent oncogenic transformation[39]. *PTEN* is a tumor suppressor gene that codifies a PI (phosphoinositide) 3-phosphatase that inhibits cellular proliferation, survival and growth by inactivating PI 3-kinase-dependent signaling. It also suppresses cellular motility through mechanisms that may be partially independent of its phosphatase activity[40].

Our results in HCC cell lines show that inactivation of these tumor suppressor genes by gene promoter hypermethylation may be implicated in HCC. However, the

genes involved are dependent on cell line characteristics, as we observed methylation of *PTEN*, *DAPK* and *p16* in HepG2 and Hep3B cell lines, while in HUH-7, the methylated genes are *DAPK* and *p16*. However, further studies, especially on the field of proteomics may be performed for the concrete conclusion about gene silencing. The fact that some of the genes we studied didn't (*p15* and *p21*) present hypermethylation may be explained by different hypothesis: they are not silenced at all or other epigenetic silencing process may be involved as Histone acetylation[50] or miRNAs[51].

We are not able to predict a pattern between etiology and methylation pattern, since we only know the etiology for the HUH-7 cell line, which has the HBV DNA integrated in its genome. Attending to the limited number of genes we studied and to the fact that we found almost the same pattern between the 3 cell lines, no conclusions can be made about the etiology and the methylation pattern.

However, as the studied genes modulated several pathways that interfere with apoptosis, cellular growth and survival or cellular motility, this study points epigenetic as a potential target for HCC therapy.

Epigenetic studies go far beyond its role on Cancer physiopathology. By detection of specific methylation patterns on many body fluids, such as blood, sputum, urine, it can be used for early tumor diagnosis and prognosis in HCC and other neoplasias[41, 42]. The effort to establish correlations between Cancer/Epigenetics and Epigenetics/Etiology is fully justified, since these can lead to a personalized diagnosis and treatment. This effort has led to the development and experiment of Epigenetic modulating drugs. We tested the effect on cell viability and death of two epidrugs,

Trichostatin A (TSA) and Decitabine (DEC), inhibitors of HDAC and DNMT, respectively[48].

Trichostatin A was able to decrease cell viability in a time and dose dependent manner (IC 50 – 200 nM) inducing cell death mainly by promoting apoptosis when compared to control (19% vs. 6%). These results are in agreement with others [48] and suggest that this drug can reactivate tumor suppressor genes previously silenced in cancer cell lines.

On the other hand, cell viability response for Decitabine was quite different. Decitabine didn't any IC 50 value. However, at the lowest concentration (1 uM) used we observe a demethylating effect suggesting that DEC in lower doses can induce TSG reactivation[49]. This lowest concentration of DEC was also responsible for a slight increase in apoptotic cells when compared to control (14, 5% vs. 6%), what is in agreement with our data that lower concentrations may be more specific for TSG reactivation.

Morphological study of HUH-7 cells treated with DEC by light microscopy showed some membrane damage not visualized in control and TSA treated cells. These membrane irregularities happen in cells with a normal nucleus, but may point to a secondary effect of DEC on these cells. DEC is a member of the group of DNMT inhibitors which is known to be not substract specific, as it inhibits other Methyltransferases besides DNMT[9]. However, no evidences were found in the literature about membrane irregularities, a fact deserving further studies.

The fact that cells treated with DEC in all concentrations registered an increase in cell viability after 48 hours may be due to the pharmacodynamics of 5'aza-2'deoxycytidine. Decitabine is a nucleoside analogue which is integrated into the

hemimethylated DNA sequence during the S phase of the cell cycle. When DNMTs are attracted to this same sequence for establishing the previous methylation pattern, they become attached to these analogues and form enzyme-DNA adducts, leading to DNMTs cell depletion[43]. This explains the lost of effectiveness of Decitabine after 48 hours, since there was only one addition of 2'Deoxy-5'azacytidine, at 0 hours. At 48 hours, it is possible that all 2'Deoxy-5'azacytidine in the wells was already metabolized.

Besides the effect of the epidrugs studied in monotherapy we also had made association studies of the drugs to test a possible synergistic effect between the two drugs. In fact, the same values of cell viability (55% at 48 hours) were achieved when a lower concentration of TSA was used (100nM) in association with DEC (1 uM) compared to TSA alone (200nM). This data is also in agreement with the literature[44]. However the efficacy of the combination is related with the schedule of drug administration as referred by other studies [44]. This is likely to be related to the complex balance between epigenetic processes, specially DNA methylation and Histone Deacetylation (Figure 3). Supporting this data is the fact that this combination led to an increase in apoptotic cells when compared to control (32% vs. 6%) and drugs in monotherapy (DEC 1uM, 32% vs. 14,5%, and to TSA alone 200 nM, 32% vs. 19%), which can be explained by a further activation of gene transcription of TSG after drug association treatment[44].

Some authors [44] have proposed hypothesis for the different results found when both kind of drugs (HDAC inhibitors and DNMT inhibitors) are added at deferred times. Our results show that lower cell viability values are reached when TSA is added 4 hours after DEC (49% at 48 hours, 40% at 72 hours). This is in agreement with the general idea that HDAC inhibitors are more effective in a hypomethylated TSG sequence[44]. On the other hand, adding DEC 4 hours later led to an increase in cell

viability that was prolonged in time (68% at 48 hours, 73% at 72 hours). This may be due to an even more unspecific role of DNMT inhibitors on a "loose" chromatin.

Finally, we tried to prove the rationale for the use of these drugs, which is the reversion of TSG hypermethylation. Our results show that there was a change in methylation pattern of *p16* gene after treatment with Decitabine 1 uM alone and Trichostatin 100nM in association with Decitabine 1 uM. These combination therapies were able to promote a decrease in methylation and the appearance of a band on the unmethylated gene. Even that these results are still scarce, they allow us to continue and deepen our studies on Epigenetics, since they prove the rationale for the use of Epigenetic modulating drugs in HCC treatment.

With this study we conclude that Epigenetics modifications are involved in Hepatocellular Carcinoma and that Epigenetic modulating drugs may have a potential a role on therapeutic approach of Hepatocellular Carcinoma

5 – Acknowledgements

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6 - References.

1. Teperino R, Lempradl A, Pospisilik JA (2013) Bridging epigenomics and complex disease: the basics. Cell. Mol. Life Sci. (2013) 70:1609–1621 DOI 10.1007/s00018-013-1299-z

2. Feinberg AP (2007) Phenotypic plasticity and the epigenetics of human disease. Nature 447(7143):433–440. doi:10.1038/ nature05919

3. Hewagama A, Richardson B (2009) The genetics and epigenetics of autoimmune diseases. J Autoimmun 33(1):3–11. doi:10.1016/j.jaut.2009.03.007

4. Kong A, Steinthorsdottir V, Masson G, Thorleifsson G, Sulem P, Besenbacher S (2009) Parental origin of sequence variants associated with complex diseases. Nature 462(7275):868–874. doi:10.1038/nature08625

5. Ling C, Groop L (2009) Epigenetics: a molecular link between environmental factors and type 2 diabetes. Diabetes 58(12):2718–2725. doi:10.2337/db09-1003

6. Schanen NC (2006) Epigenetics of autism spectrum disorders. Hum Mol Genet 15 (Spec No 2):R138–R150. doi:10.1093/hmg/ ddl213

7. Huang J (2009) Current progress in epigenetic research for hepatocarcinomagenesis. Science in China Series 52:31-42 doi:10.1007/s11427-009-0014-7

8. Lorella Vecchio a, Paul Faustin Seke Etet b, Maulilio John Kipanyula, Mauro Krampera, Armel Hervé Nwabo Kamdje (2013) Importance of epigenetic changes in cancer etiology, pathogenesis, clinical profiling, and treatment: What can be learned from hematologic malignancies? Biochimica et Biophysica Acta 1836:90–104

9. Mund C, Lyko F (2010) Epigenetic cancer therapy: Proof of concept and remaining challenges. Bioessays 32:949-957 doi: 10.1002/bies.201000061

10. Okano M, Bell DW, Haber DA, Li E. 1999, DNA methyltranferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 99:247-57

11. M. Hattori, H. Sakamoto, K. Satoh, T. Yamamoto, DNA demethylase is expressed in ovarian cancers and the expression correlates with demethylation of CpG sites in the promoter region of c-erbB-2 and survivin genes, Cancer Lett. 169 (2001) 155–164

12. A.K. Maunakea, R.P. Nagarajan, M. Bilenky, T.J. Ballinger, C. D'Souza, S.D. Fouse, B.E. Johnson, C. Hong, C. Nielsen, Y. Zhao, G. Turecki, A. Delaney, R. Varhol, N. Thiessen, K. Shchors, V.M. Heine, D.H. Rowitch, X. Xing, C. Fiore, M. Schillebeeckx, S.J. Jones, D. Haussler, M.A. Marra, M. Hirst, T. Wang, J.F. Costello, Conserved role of intragenic DNA methylation in regulating alternative promoters, Nature 466 (2010) 253–257

13. Liu W, Shi Y, Peng, Y, Fab J (2012) Epigenetics of hepatocelular carcinoma: a new horizon, Chinese Medical Journal 125(13):2349-2360.

14. Mund C, Lyko F (2010) Epigenetic cancer therapy: Proof of concept and remaining challenges. Bioessays 32:949-957 doi: 10.1002/bies.201000061

15. Rajagopal N, Clifford S, Cressman E (2008) Molecular Mechanisms of Hepatocellular Carcinoma, Hepatology DOI: 10.1002hep.22580

16. Feinberg AP, Gehrke CW, Kuo KC, Ehrlich M. 1988. Reduced genomic 5-methylcitosine content in human colonic neoplasia. Cancer Res 48: 1159-61.

17. Kondo Y, Shen L, Suzuki Kurokawa T. 2007, Alterations of DNA methylation and histone modifications contribute to gene silencing in hepatocellular carcinoma. Hepatol Res 37:974-83.

18. Streseman C, Lyko F. 2008. Modes of action of the DNA Methyltransferase inhibitors azacytidine and decitabine. Int J Cancer 123: 8-13.

19. Marks PA, Breslow R. 2007. Dymethilsulphoxide to Vorinostat: development of this Histone deacetylase inhibitor as an anticancer drug. Nat Biotechnolo 25: 84-90.

20. Jemal A. Bray F. Center MM, Ferlay J. Ward E, Forman D. 2011. Global cancer statistics, CA Cancer J Clin. 61: 69-90.

21. Mary Maluccio, Anne Covey, 2012. Recent Progress in Understanding, Diagnosing and Treating Hepatocellular Carcinoma. Cancer Journal for Clinician doi:10.3322

22. Abou-Alfa GK, Schwartz L, Ricci S. 2006. Phase II Study of sorafenib in patients with advanced hepatocellular carcinoma. J Clinical Oncology. 24: 4293-4300.

23. Llovet JM, Ricci S, Mazzaferri V. SHARP Investigators Study group. 2009. Sorafenib in advanced hepatocellular carcinoma. N Eng J Med. 359:378-390.

24. Cheng AL, Kang Y, Chen Z. 2009. Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomized, double blind, placebo controlled trial. Lance Oncol. 10: 25 – 34.

25. Pawlik TM, Reyes DK, Cosgrove D, Kamel IR, Bhagat N, Geschwind JF. 2011. Phase II trial of sorafenib combined with concurrent transarterial chemoembolization with drug eluting beads for hepatocellular carcinoma. J Clinical Oncol. 29: 3960-3967.

26. Huang J, 2009. Current progress in epigenetic research on hepatocarcinomagenesis. Science in China Series C: Life Sciences. 52: 31-42.

27. Ushijima T, Morimura K, Hosoya. 1997- Establishment of methylation-sensitive-representational difference analysis and isolation of hypo- and hypermethylated genomic fragments in mouse liver tumors. Proc Natl Acad Sci USA. 94(6): 2284-2289.

28. Calvisi DF, Ladu S, Gorden A. 2007. Mechanistic and prognostic significance of aberrant methylation in the molecular pathogenesis of human hepatocellular carcinoma. J Clin Invest. 117(9): 2713-2722.

29. Nambu S, Inoue K, Saski H. Site-specific hypomethylation of the *c-myc* oncogene in human hepatocellular carcinoma. Jpn J Cancer Res, 1987, 78(7): 695－704

30. Chaubert P, Gayer R, Zimmermann A, et al.Germ-line mutations of the *p16INK4*(*MTS1*) gene occur in a subset of patients with hepatocellular carcinoma. Hepatology, 1997, 25(6): 1376－1381

31. Narimatsu T, Tamori A, Koh N, et al. *p16* promoter hypermethylation in human hepatocellular carcinoma with or without hepatitis 29 virus infection. Intervimlogy, 2004, 47(1): 26－31

32. Zhang Y J, Ahsan H, Chen Y, et al.High frequency of promoter hypermethylation of *RASSF1A* and *p16* and its relationship to aflatoxin B1-DNA adduct levels in human hepatocellular carcinoma. Mol Carcinog, 2002, 35(2): 85－92

33. Oh B K, Kim H, Park H J, et al. DNA methyltransferase expression and DNA methylation in human hepatocellular carcinoma and their clinicopathological correlation. Int J Mol Med, 2007, 20(1): 65－73

34. Lei W, Zhang K, Pan X, Hu Y, Wang D, Yuan X. 2010. Histone deacetylase 1 is required for transforming growth factor beta1-induced epithelial mesenchymal transition. Int J Biochem Cell Biol. 42: 1489-1497.

35. Choi HN, Bae JS, Jamiyandorj U; Noh SJ, Park HS, Jang KY. 2011. Expression and role of SIRT1 in hepatocellular carcinoma. Oncol Rep. 26: 503-510.

36. Martin M, Herceg Z, 2012. From hepatitis to hepatocellular carcinoma: a proposed model for cross-talk between inflammation and epigenetic mechanisms. Genome Medicine. 4:8

37. Serrano M, Hannon GJ, Beach D: A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature* 1993, 366:704-707.

38. Deiss LP, Feinstein E, Berissi H, Cohen O, Kimchi A: Identification of a novel serine/threonine kinase and a novel 15-kD protein as potential mediators of the gamma interferon-induced cell death. *Genes Dev* 1995, 9:15-30.

39. Raveh T, Droguett G, Horwitz MS, DePinho RA, Kimchi A: DAP kinase activates a p19ARF/p53-mediated apoptotic checkpoint to suppress oncogenic transformation. *Nat Cell Biol* 2001, 3:1-7.

40. Nick R. Leslie and C. Peter Downes, 2004. Biochem J. 2004 August 15; 382(Pt 1): 1–11.

41. Laird, P.W., 2003. The power and the promise of DNA methylation markers. Nat. Rev., Cancer 3, 253–266.

42. Shi, H., Wang, M.X., Caldwell, C.W., 2007. CpG islands: their potential as biomarkers for cancer. Expert Rev. Mol. Diagn. 7, 519–531.

43. Creusot, F., Acs, G., Christman, J.K., 1982. Inhibition of DNA methyltransferase and induction of Friend erythroleukemia cell differentiation by 5-azacytidine and 5-aza-2′- deoxycytidine. J. Biol. Chem. 257, 2041–2048

44. Cameron, E.E., Bachman, K.E., Myohanen, S., Herman, J.G., Baylin, S.B., 1999. Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. Nat. Genet. 21, 103–107.

45. Herman JG, Graff JR, Myohanen S, Nelkin BD and Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proceedings of the National Academy of Sciences of the United States of America 1996; 93:9821-9826

46. O'Brien J, Wilson I, Orton T and Pognan F. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. European journal Biochemistry, 2000; 267:5421-5426

47. Aubry JP, Blaecke A, Lecoanet-Henchoz S, Jeanin P, Herbault N, Caron G. Annexin-V used for measuring apoptosis in the early events of cellular toxicity. Cytometry 1999; 37:197-204.

48. João A. Carvalho, Filipa Carvalho; André Ribeiro; Vera Alves; Ana Cristina Gonçalves; Ana Bela Sarmento-Ribeiro' Epigenetics: a new therapeutic approach in Chronic Lymphocytic Leukemia. Master thesis in Medicine, FMUC 2012.

49. Wong YF, Jakt LM, Nishikawa, Prolonged treatment with DNMT inhibitors induces distinct effects in promoters and gene bodies, PLOS onec 2013; 6.

50. Chen YX, Fang JY, Lu J, Qiu DK, Regulation of histone acetylation on the expression of cell cycle-associated genes in human colon cancer cell lines, Zhonghua Yi Xue Za Zhi. 2004 Feb 17;84(4):312-7

51. Yu W, Gius D, Onyango P, Muldoon-Jacobs K, Karp J, Feinberg AP, Cui H, Epigenetic silencing of tumour suppressor gene p15 by its antisense RNA, Nature. 2008 Jan 10;451(7175):202-6. doi: 10.1038/nature06468