



UNIVERSITÀ DEGLI STUDI DI TRIESTE

DIPARTIMENTO DI SCIENZE DELLA VITA

SCHOOL OF MOLECULAR BIOMEDICINE

The Importance of Death Receptor Pathway in Hepatocellular Carcinoma and Its Potential in the Development of Oligonucleotide Nanomedicine

*A thesis submitted for the degree of
Doctor of Philosophy in Molecular Biomedicine (PhD)*

- Cycle XXXI -

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2017/2018



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LIST OF ABBREVIATIONS

HCC: Hepatocellular carcinoma. **NAFLD:** Non-Alcoholic Fatty Liver Disease. **NASH:** Non-Alcoholic Steatohepatitis. **BCLC:** Barcelona Clinic Liver Cancer. **TNF- α :** Tumor Necrosis Factor α . **TNFR1:** Tumor Necrosis Factor Receptor 1. **DR3:** Death Receptor 3. **TRAIL-R1:** TNF-related apoptosis-inducing ligand receptor 1. **PUMA:** p53 up-regulated modulator of apoptosis. **NF- κ B :** Nuclear Factor kappa-light-chain-enhancer of activated B cells. **TP53:** Tumor Protein p53. **FADD:** Fas-associated protein with death domain. **DISC:** Death Inducing Signal Complex. **ASO:** Anti-sense oligonucleotides. **RNAi :** RNA interference. **siRNA:** small interfering RNA. **shRNA:** short hairpin RNA. **miRNA:** micro RNA. **DICE:** Death induced by the Fas/FasL elimination. **MOMP:** Mitochondrial outer membrane permeabilization. **SELEX:** Systematic Evolution of Ligands by Exponential Enrichment.

SUMMARY

The importance of cell death receptor pathway in hepatocellular carcinoma and its potential in the development of oligonucleotide nanomedicine

Rusdina Bte Ladju

Hepatocellular carcinoma (HCC; primary liver cancer) is one of the most common cancers with high mortality rate worldwide. Hepatocarcinogenesis is triggered by multiple factors, such as cancer etiologies, treatment options, host factors, and life style. It is a complex progressive disease involving a sustained inflammatory damage, fibrosis, and cirrhosis. Numerous numbers of intrinsic and extrinsic factors lead to the alteration of various molecular and cellular events, compromising the balance between survival and apoptotic signals in the cells.

Fas (CD95, Apo-1, TNFRSF6) is a member of cell death receptor in tumor necrosis factor receptor superfamily. It has major function in inducing apoptosis upon engagement by its natural ligand FasL (CD95L, CD178, TNFSF6). In cancer, however, the role of Fas/FasL axis is extensive, including in non-apoptotic and cancer-promoting functions. In this study, we showed the importance of Fas/FasL axis in HCC. By using different experimental settings, we demonstrated that Fas and FasL expressions were associated with p53/PUMA intrinsic apoptosis pathway, tumor growth, inflammation, and cancer stemness.

In *in vivo* study by utilizing human HCC clinical samples and normal liver, the presence of Fas and FasL were analyzed. The expression of Fas was found significantly higher in HCC nodules compared cirrhosis and normal donor livers, while FasL was high in both HCC and cirrhosis compared to normal. In contrast, PUMA expression was dysregulated from normal to cirrhosis and to be lowest in HCC.

In *in vitro* study by using hepatic normal and cancer cell lines, the modulation of Fas were analyzed by two methods: 1. extrinsic apoptotic pathway activation by anti-Fas in both acute and chronic exposure, and 2. gene silencing of Fas. By two methods, Fas modulation was found to be diverse for cancer cells compared to normal cells. In normal cells, Fas was shown to be correlated mainly in pro-apoptotic pathway and cells growth, while in cancer cells, in addition to apoptosis and

cells growth, Fas is also related to cancer stemness. In particular, for acute anti-Fas exposure, modulation of transcription factors, apoptotic and inflammation genes were strongly noticed.

Due to the significance of Fas in HCC, the last part of the thesis described the development of potential oligonucleotide aptamers against Fas as a molecular probe for HCC. Aptamers are single-stranded RNA or DNA oligonucleotides with low molecular weight that specifically and efficiently bind to a target molecule. The screening and evaluation of DNA aptamers recognizing Fas were developed through a cell-based Systematic Evolution of Ligands by Exponential Enrichment (SELEX) in Fas+ and Fas- HCC cell lines. The aptamers candidate pools by SELEX were cloned and will be subjected to sequencing for subsequent bioinformatics screening and validation assay. Future Fas aptamers can be potentially used for theranostics (therapy and diagnostic) approach, biosensors, and drug nano-delivery system.

In summary, our data indicated the importance of extrinsic cell death receptor pathway Fas/FasL axis in HCC. This study contributes in the understanding and the development of potential future therapy against HCC.

Chapter 1

GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

1.1. HEPATOCELLULAR CARCINOMA

Hepatocellular carcinoma (HCC) is one of the most common cancers with an incidence of approximately 782,500 new cases per year and the second leading cause of cancer-related death worldwide (Figure 1.1) ¹. The incidence rate and the burden of HCC are increasing with high substantial morbidity and mortality in several regions in Europe, Africa, and the United States, contrary with decreasing trend in an endemic area such as East Asia ^{2,3}. HCC is the most frequent primary liver cancer, which represents around 90% of all cases occurring worldwide ⁴.

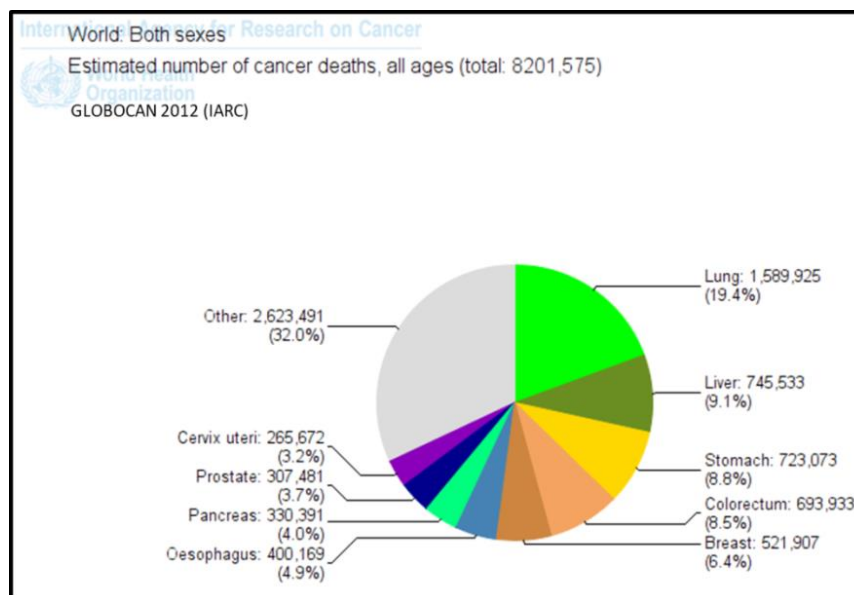


Figure 1.1. Number of cancer-related death worldwide

1.1.1 EPIDEMIOLOGY

Variations among geographic region, ethnicity, age, and gender are several interesting epidemiologic features of the HCC. Incidences rate of HCC based on age-standardized were high in Asia and Africa (Figure 1.2)⁵. Men have a high prevalence of HCC than women with 462,400:185,800 new cases per year in developing country⁶. Overall, the geographic distribution of HCC accompanies by its etiology and major risk factors. The incidence of HCC is expected to increase

in the future, especially in America and northern and central Europe, where diabetes, obesity, and alcohol abuse represent the major risk factors⁷⁻⁹.

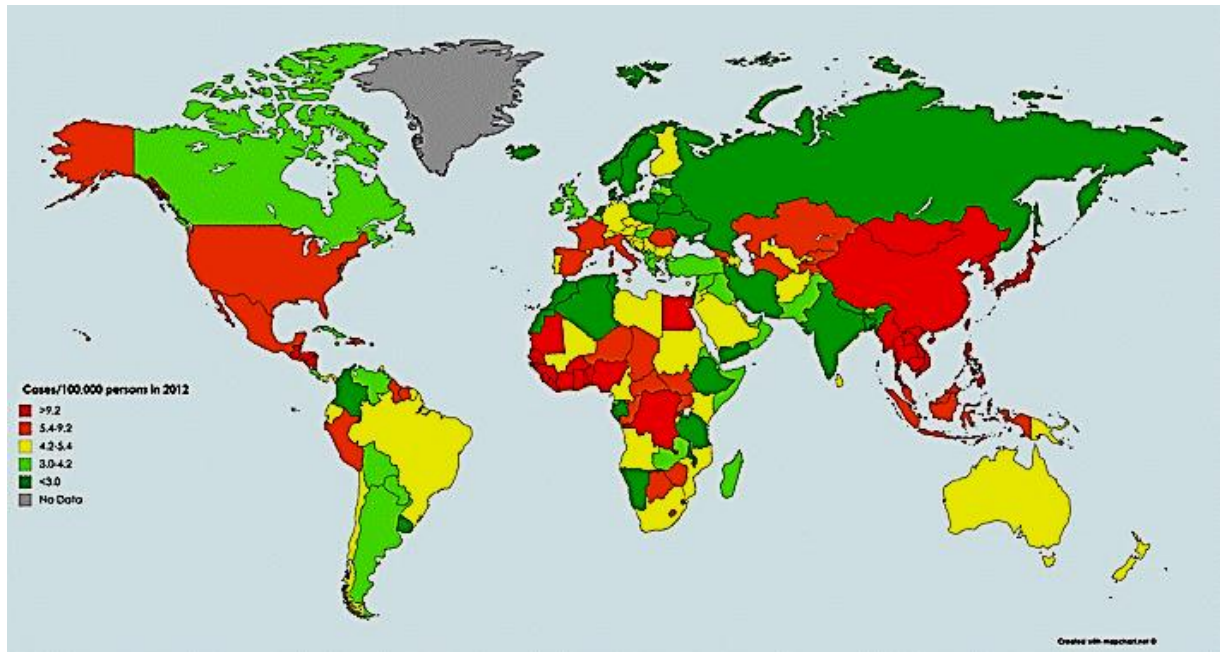


Figure 1.2. Geographic distribution of incidence rates of liver cancer

1.1.2. ETIOLOGIES AND RISK FACTORS

Risk factors and etiological agents that responsible for the development of HCC are identified and characterized as the major risk factors which commonly leading to HCC development. The well-known risk factors and etiologies for HCC are reported in Table 1.1. Well-known risk factors and etiologies of HCC vary depending on geographic region, lifestyle, and advanced medical precaution.

Hepatitis B and C are the main etiological factors that have significant contributing to hepatocellular carcinoma. Chronic hepatitis B infection was responsible for 44% of HCC cases, and hepatitis C was caused by 21% of the cases worldwide. The highest cases of hepatitis B incidence were in Asia. In Western, Central, and Eastern Europe and North America, NAFLD/NASH, obesity, and excessive alcohol consumption were responsible for a higher

percentage of HCC cases that contrary in Asia and Africa where tobacco smoking is the most contributable risk factor of HCC ^{10,11}. HCC caused by viral or metabolic etiologies has a high risk of recurrent de novo, which develops into incurable and more advanced staging even after complete tumor resection or ablation³.

Table 1.1. Risk factors of hepatocellular carcinoma

Risk factors	Ref
Age	6,9,12–14
Gender	6,9,12–14
Ethnicity	6,9,12,13
Chronic Hepatitis B Virus/Hepatitis C Virus infection	6,12
Alcoholism	6,12
Aflatoxin-B1 intoxication	6,12
Obesity	6,12
Type II diabetes	6,12
Metabolic syndrome	6,12
Genetic predisposition	12
Smoking	12
Fatty liver Non-alcoholic steato-hepatitis	12
Hypothyroidism	12
Hemochromatosis	12
Tyrosinemia, Galactosidemia, Fructosemia	12
Glucose overload	12
Alpha 1 anti-trypsin deficiency	12
Anabolizing hormones	12

1.1.3. HEPATOCARCINOGENESIS

HCC is a multifactorial disease caused by a variety of risk factors with several cellular phenomena including hypoxia, inflammation reaction, oxidative stress, and tumor microenvironment. The molecular mechanism of liver carcinogenesis involved multiple endogenous and exogenous genetic alterations¹⁵. Hepatocarcinogenesis is a deliberate and complex multistep process associated with somatic genomic alterations in producing cellular intermediates that progress into hepatocellular carcinoma¹⁶. Development of HCC is a combination of continuous inflammatory damage, necrosis, and fibrotic deposition. The pre-neoplastic stage is a long process which takes time 10 to 30 years. During the stage, phenotypically altered hepatocytes are results from DNA methylation alteration, pathogenic agent's reaction, and point mutation or loss of heterozygosity, which are in part through epigenetic mechanisms and modification that leads to the development of dysplastic hepatocytes in foci and nodules. Aberrant and dysplastic hepatocytes are related to the accumulation of permanent structural alteration and changes in genes and chromosomes¹⁷. The alterations in the malignant phenotype are often distinct, suggesting heterogeneity on the genomic level (Figure 1.3)^{17,18}.

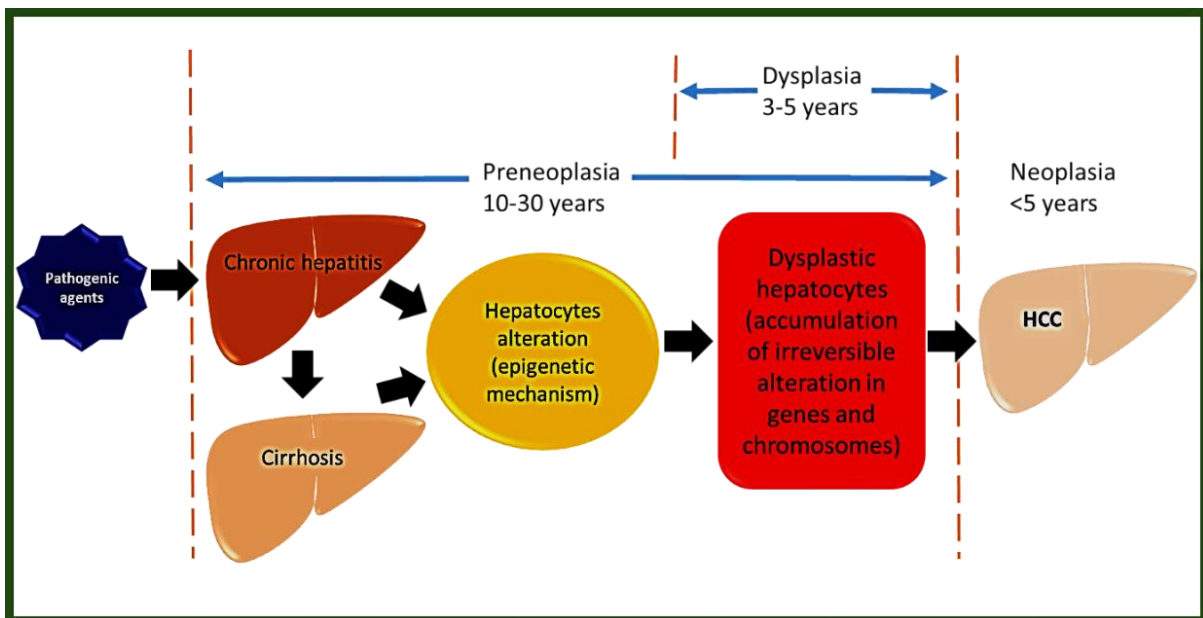


Figure 1.3. Hepatocarcinogenesis: the development of HCC in human

1.1.4. DIAGNOSIS

Recently, novel improvement has emerged in the field of HCC compared with several years ago. In developed countries, 30-60% of HCC cases are feasible and diagnosed at the early stage, which enables for a curative treatment. In contrast with developing countries, most of the cases were diagnosed at a late stage of HCC, where there are fewer possibilities to do the curative treatment. Diagnosis of HCC is an important and critical phase related to the survival and prognostic of the patients. The diagnosis of HCC is based on non-invasive criteria and pathological criteria (Figure 1.4)¹⁹.

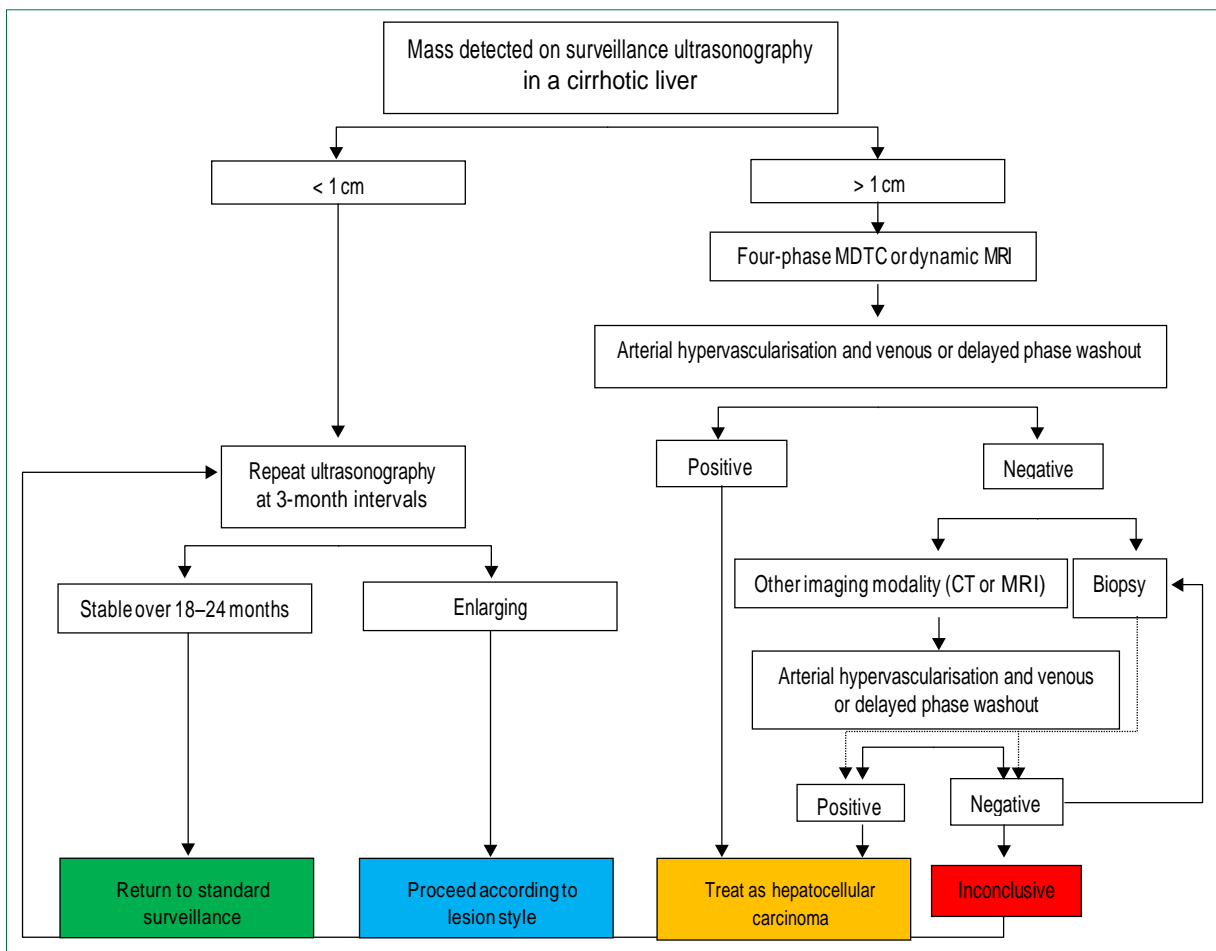


Figure 1.4. Diagnostic algorithm for HCC¹⁹

1.1.5. CURRENT TREATMENTS AND OBSTACLES

The success of HCC treatment primarily depends on the time of diagnosis. Early diagnosis is crucial for a favorable prognosis since curative therapies options, such as local radiofrequency ablation and surgical intervention (liver transplantation and liver resection), have much higher efficacy in the very early and early-stage HCC as compared to later stages^{9,20}. Liver transplantation can be the best treatment for HCC with a low risk of recurrence. However, due to the disparity of liver donor resources and the increasing number of patients, it is suggested as a second-line treatment only in case of relapse or liver failure after liver resection and ablation therapy²¹. Patients in later stages HCC (intermediate and advanced), can receive palliative treatments such as chemoembolization and kinase inhibitors, while for patients in the terminal stage can only receive best supporting care²². Figure 1.5 shows the most recently updated version of the Barcelona Clinic Liver Cancer (BCLC) system that has been extensively validated for the staging and treatment strategy of HCC¹⁹.

Nevertheless, tumor recurrence after percutaneous ablation or liver resection treatment can be a problem also in the early stages HCC. The probability of 5 years HCC recurrence is around 80% after liver resection²³ and 62% after liver ablation²⁴. Furthermore, palliative treatments for intermediate and advanced stages often have an unfavorable outcome due either to drug side effects or drug resistance. A recent study done by Njei *et al.* showed that only 46.2% of HCC cases are diagnosed at an early stage where most of the cases do not receive curative therapy²⁵.

Based on this evidence, HCC treatment options are still hampered by many obstacles. Therefore, the development of early diagnostic tools and new therapeutic approaches will be crucial to improving the survival rate and life quality of the patient.

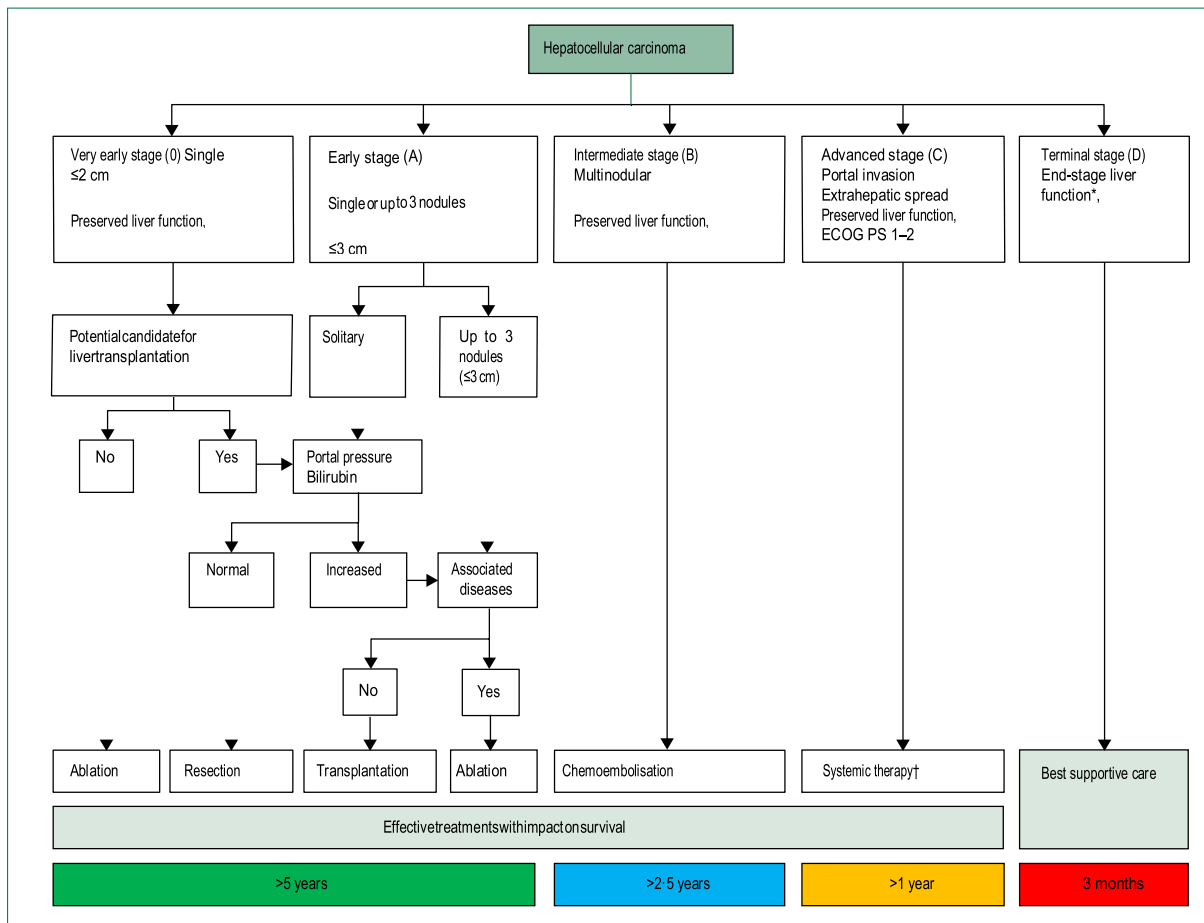


Figure 1.5. Barcelona Clinic Liver Cancer (BCLC) staging and treatment strategy¹⁹

1.1.6. PREVENTION STRATEGY

Early detection and prevention of HCC progress is the most effective and crucial strategy to reduce the mortality rate and improve patient prognosis. Since the number of incidences is keep increasing, new strategies are needed for the enhancement and implementation of preventive approaches to decrease the burden of HCC. Various types of medical intervention must be incorporated for the prevention achievement. There are three main HCC-preventive strategies and interventions for targeting specific clinical contexts of HCC (Figure 1.6).

The primary prevention strategies focus on lifestyle modification, preventing exposure to cancer predisposing factors, and eliminating the etiology of chronic Hepatitis B/C virus infection

by routine universal vaccination. The secondary prevention comprises of regular HCC screening and treatment based on the etiology-specific in high-risk population and risk factor modification. These include chemo-prevention and control the risk factor of metabolic disease risk factors ^{3,26}.

Potential HCC chemo-preventions are including anti-viral therapy, anti-inflammatory, immune-modulatory, statins, anti-diabetic drug, anti-fibrotic, nutritional substance, other metabolic disease treatment, and molecular targeted interventions ^{3,27}. Development of new HCC biomarkers, integrative scores, and imaging modalities may improve the sensitivity and specificity of HCC screening and tumor detection. Combinations of clinical and molecular HCC risk scores and HCC screening the individual patients are more cost-effective and optimize the limited medical resources.

Individual risk prediction is estimated to overcome the challenge by supporting personalized chemoprevention, targeting high-risk patients for fastidiousness HCC prevention, and significantly improving the quality of life and prognosis of HCC patient³. The tertiary prevention is focusing on the post-treatment monitoring and continuation of antiviral in established HCC^{3,26}. Implementation and clinical assessment of HCC preventive approaches, including HCC screening in a high-risk population and potential chemo-prevention intervention, will not be achievable without individual risk-based personalized approaches. Classification of the potential candidate of chemo-preventive targets at risk of cancer development is assists and categories based on wide-ranging, multi-omics, and multi-cell types characterization of liver disease and tissue microenvironment³. Therefore, the prevention of HCC development is the most impactful and effective strategy to prevent HCC development and improve HCC prognosis.

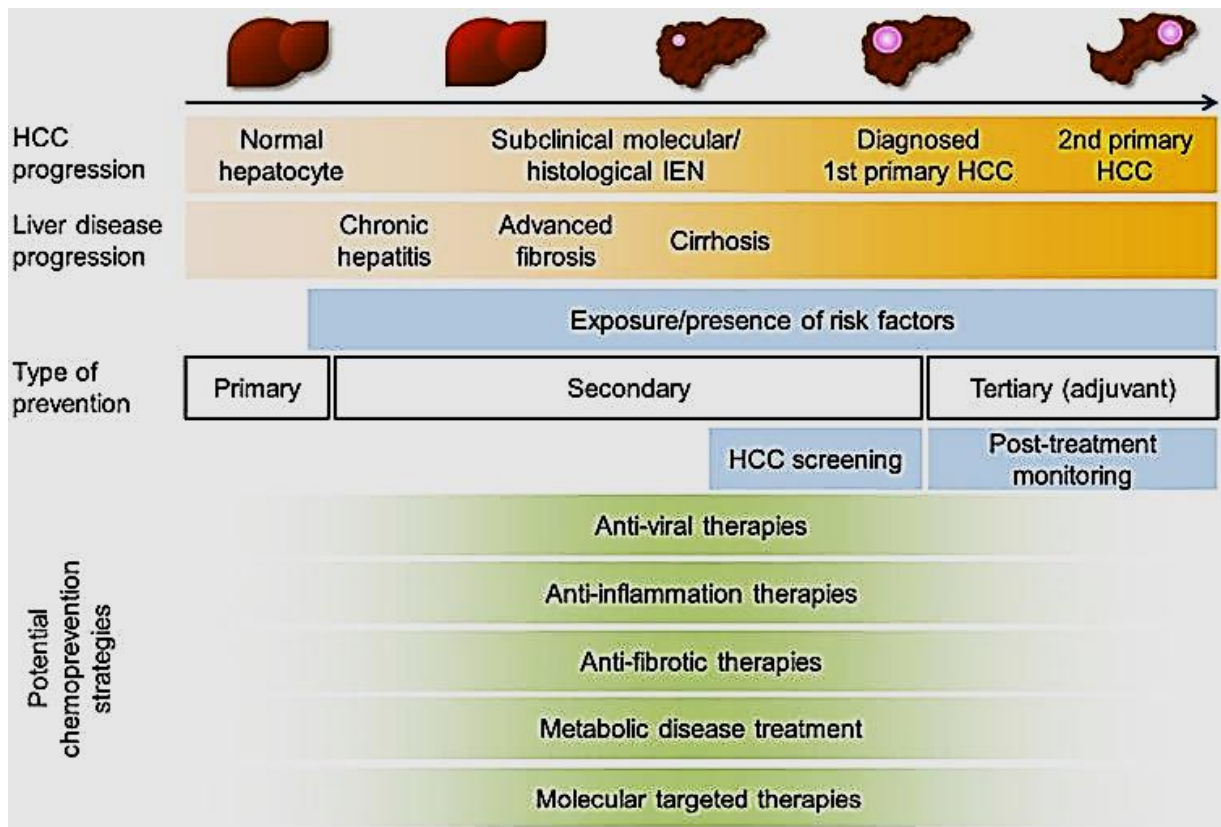


Figure 1.6. HCC preventive strategy and interventions ³

1.2. CELL DEATH PATHWAY IN HCC

Apoptosis is an orchestrated cellular process, known as a type I programmed cell death that is important in balancing the cell survival and cell death in the development and cellular homeostasis. Apoptosis described by several morphological changes in the structure of the cells. The cell membrane is bleb and becomes *pyknosis* as the result of the chromatin condensation. DNA fragmentation followed by *karyorrhexis* and apoptotic body formation that will rapidly be engulfed by phagocytes (Figure. 1.7).

The apoptosis process has minimal damage to the surrounding interstitial tissues because of the absence of inflammatory reaction due to no anti-inflammatory cytokines production and release of cellular constituents. The mechanisms of apoptosis encompassing an energy-dependent biochemical cascade of molecular events that are highly sophisticated and complex,

which occurs in physiological and pathological conditions^{28–30}.

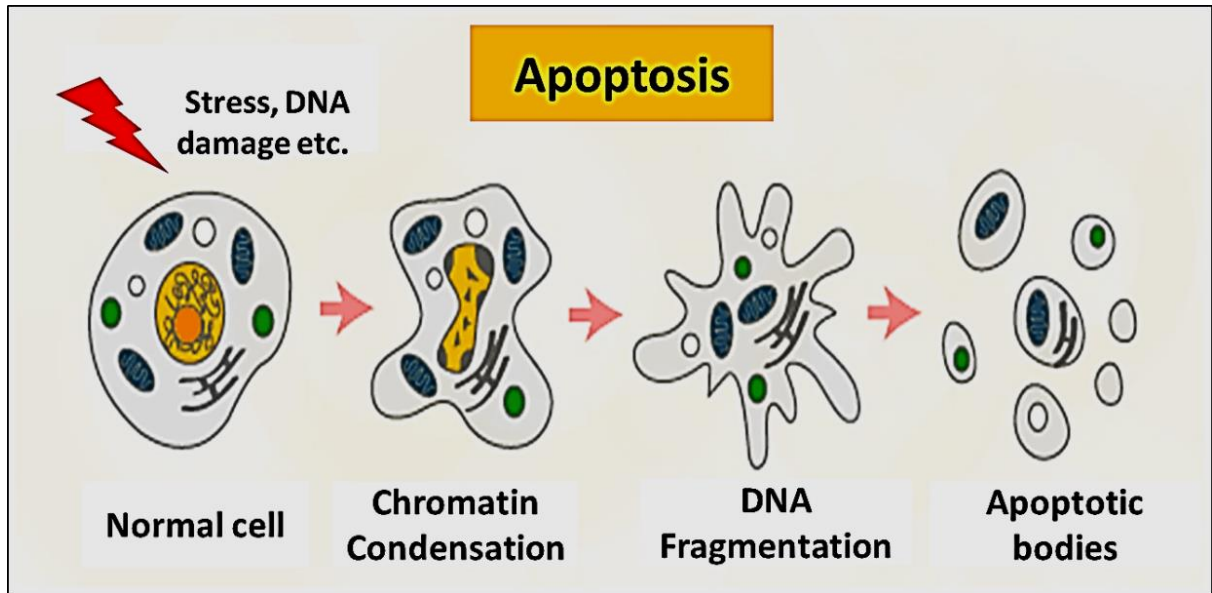
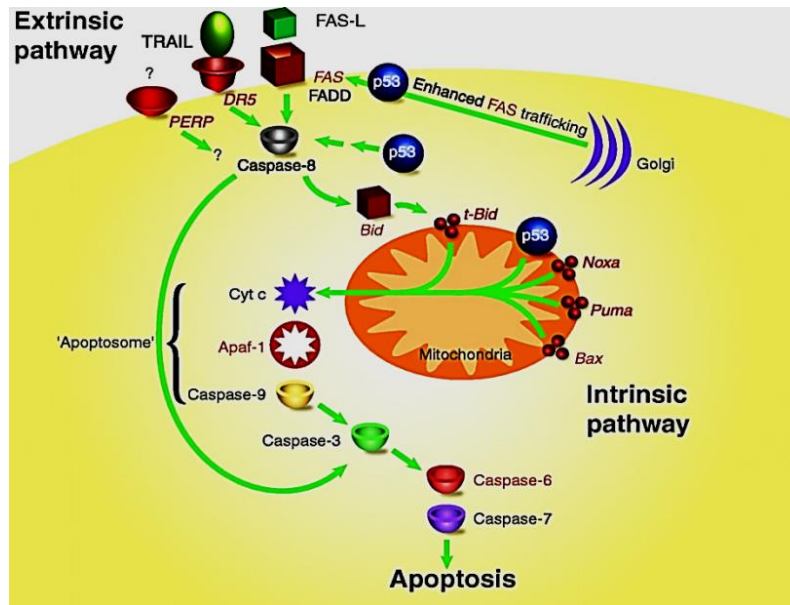


Figure 1.7. Cell morphological changes in apoptosis

Cellular homeostasis and growth-control mechanism are interconnected with apoptosis. Failure of apoptosis or inactivation of apoptosis function resulting the accumulation of damaged cells that fundamental to the development of various forms of cancer. Therefore, apoptotic-resistance might be the main key factor and essential feature of cancer development^{28,31}.

Since the mechanism of apoptosis involves multi-steps and multi-pathway, defects, or malfunction at the pathway will disturb the apoptosis process and lead the affected cells into malignant transformation. Down-regulation or mutation of pro-apoptotic proteins and the expression or up-regulation of anti-apoptotic proteins in tumor cells can lead to apoptosis resistance^{32,33}. Apoptosis can be triggered and activated either through the death-receptor or extrinsic pathway and the mitochondrial or intrinsic pathway (Figure 1.8). Both pathways will activate their specific effector caspases that cleave pro-apoptotic substrate leading to biochemical and morphological changes^{33,34}.



*Figure 1.8. Apoptosis pathway*³⁵

1.2.1. EXTRINSIC PATHWAY

Apoptosis that initiates through the extrinsic signaling pathway is triggered by transmembrane death receptor and ligand interactions^{29,36}. Death receptors are members of tumor necrosis factor (TNF) superfamily are characterized by a death domain (cytoplasmic region) that plays a crucial role in transferring and initiating the death signal from the cell surface into the intracellular signaling cascades when engaged by its homologous trimeric ligands^{37,38}. The corresponding death receptors and its ligands include Fas or CD95/FasL or CD95L, TNFR1 (tumor necrosis factor (TNF) receptor 1)/TNF- α , DR3 (death receptor 3) also known as TRAMP/TL1A, TRAIL-R1 (TNF-related apoptosis-inducing ligand receptor 1) also known as DR4 (death receptor 4) /Apo2L, TRAIL-R2 (TNF-related apoptosis-inducing ligand receptor 2) also called as DR5 (death receptor 5)/Apo2L (Figure 1.9)³⁹.

Among all of the death receptors, Fas is a prototype death receptor and the key component of the extrinsic death pathway characterized by the existence of 80 amino acid death

domain in the cytoplasmic tail ^{40,41}. The binding of Fas and its cognate Fas ligand results in the binding of the adapter protein FADD to form the death-inducing signaling complex (DISC) that activating the procaspase-8 via dimerization of the death effector domain. The execution phase of the apoptosis trigger due to the activation of caspase-8 ^{38,39,42,43}.

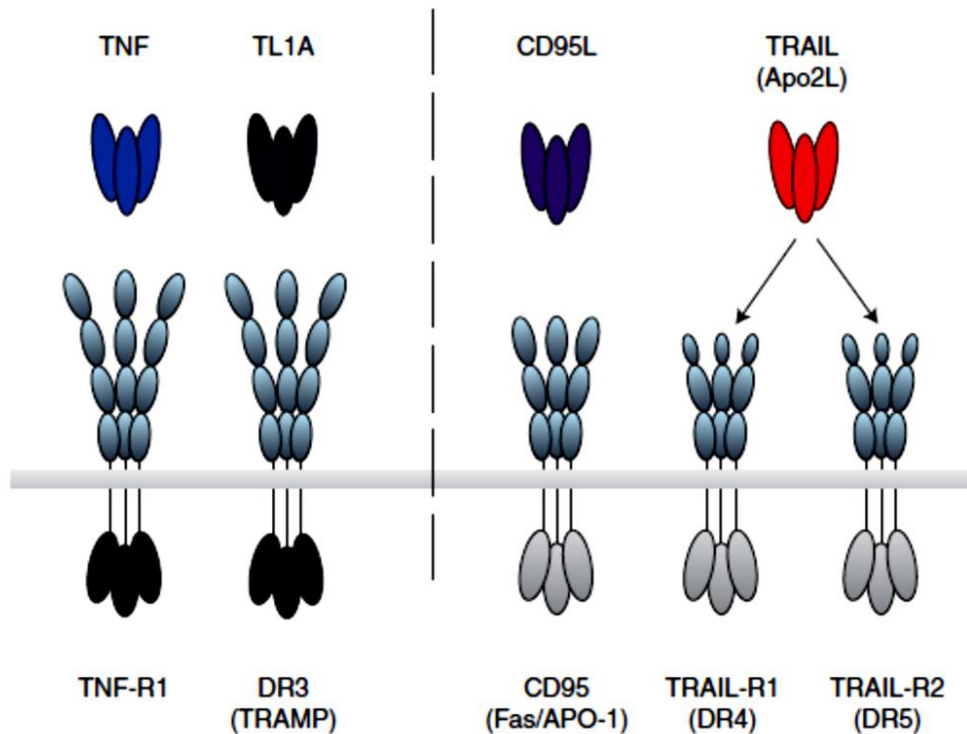


Figure 1.9. Death receptors and their ligands³⁹

Death receptors are mainly recognized as apoptosis inducers in normal conditions. However, in a pathologic conditions such as cancers, death receptors also fulfill a variety of non-apoptotic functions ⁴².

1.2.2. INTRINSIC PATHWAY

The mitochondrial pathway of apoptosis implicates various stimuli that produce intracellular signals directly on target in either a positive or negative way. Stimuli increase the mitochondrial permeability transition cause changes in the inner mitochondrial membrane. The intrinsic pathway initiated by p53 stimulation. Activation of p53 triggers the pro-apoptotic proteins, which cause mitochondrial outer membrane permeabilization (MOMP), leading to the release of cytochrome *c* from mitochondria into the cytoplasm. The cytochrome *c* binds with Apaf-1 to form the apoptosome complex in the cytoplasm. The apoptosome complex mediates the conversion of inactive pro-caspase-9 into active caspase-9. The caspase-9 is important to activate the caspase-3 that leads to caspase cascade, resulting in apoptosis (Figure 10)⁴⁴.

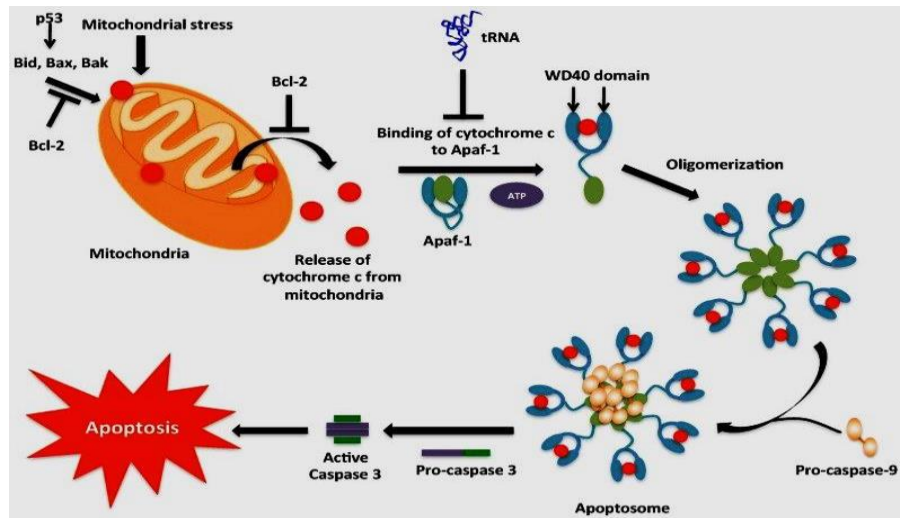


Figure 1.10. Mitochondrial pathway⁴⁴

Apoptosis activation by negative feedback occurs due to the absence of certain factors that can lead to failure of death suppression. tRNA is one of the factors that may interact with cytochrome *c* and prevent its binding to Apaf-1. The tRNA and cytochrome *c* may lead to failure apoptosis activation. Radiation, toxins, hypoxia, hyperthermia, and free radicals are stimuli that work in positive feedback^{29,44}.

1.2.3. FAS DEATH RECEPTOR

Fas (also known as TNFRSF6/CD95/APO-1), is a transmembrane protein that plays an important role in cellular homeostasis and regulation of the immune system. Fas death receptor induces apoptosis upon binding to its cognate ligand FasL/CD95L. Fas death receptor is the key component of the extrinsic apoptosis⁴⁰. The Fas gene is located at chromosome 10q24.1 (Figure 12) that consists of 9 exons and 8 introns.

Aberrant expression of the Fas gene has been discovered in immune-related disease in humans. Fas gene also reported in promoting carcinogenesis due to A to G transition at nucleotide position -670 (rs1800682), located within the signal transducer and activator of transcription (STAT-1), which can influence the Fas expression and deregulate cell death signaling^{45,46}. Fas gene polymorphism and mutation are probably related to pathological conditions. Fas inactivation or dysfunction may be involved in malignant transformation. Polymorphism of the Fas gene at nucleotide position rs1800682A/G may be associated with hepatocellular carcinoma susceptibility⁴⁷. Mutation in the Fas gene (Figure 13) caused premature termination of the Fas gene alleles in leukemia, resulting aberrant expression of Fas⁴⁸.

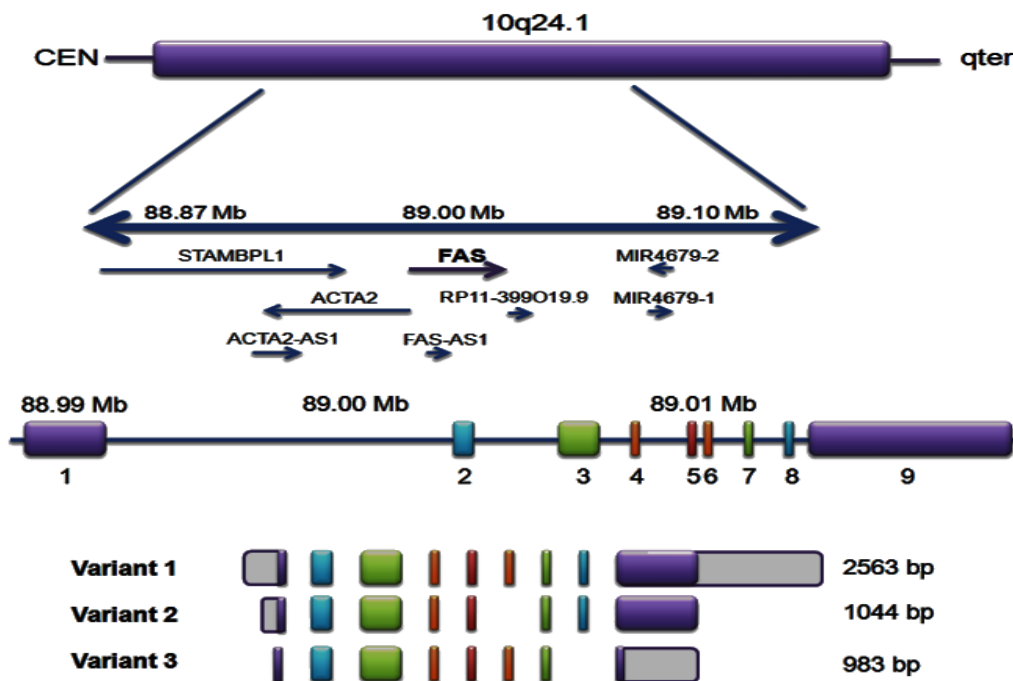


Figure 1.11. Fas gene (Atlas genetic oncology)

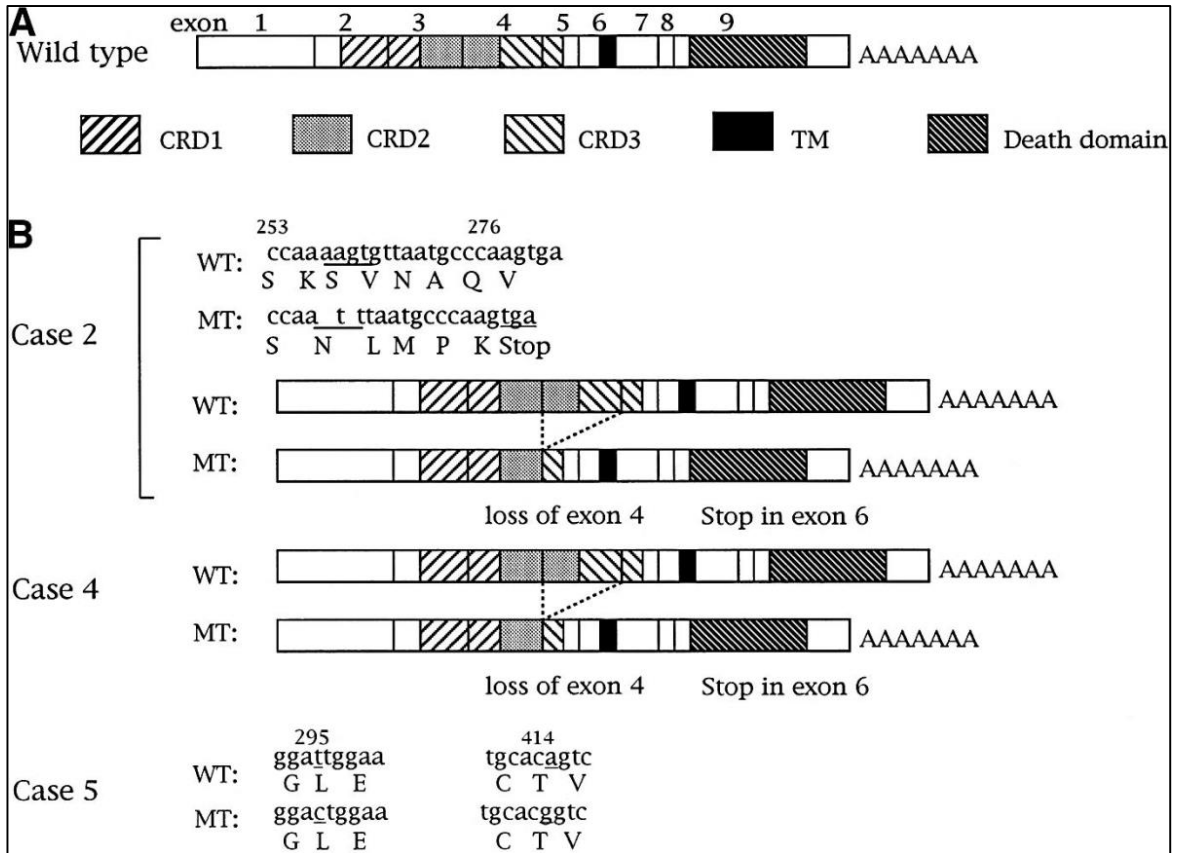


Figure 1.12. Fas gene mutation in leukemia⁴⁸

1.2.4. FAS PROMOTE TUMOR GROWTH AND CANCER STEMNESS

Fas death receptor is important and well-function as an apoptotic inducer in a normal condition. However, in pathologies condition such as inflammatory disorders and cancers, the non-apoptotic function was dominant due to mutation of Fas death receptor or its ligand⁴⁹. Fas and FasL play a pivotal role in cancer cells. Fas and FasL promoting tumor growth, cancer invasiveness, and increase the number of cancer stem cells (Figure 14). Fas stimulation's protecting cancer cells from cell death and increases the stemness of cancer cells by inducing a STAT1 dependent type 1 interferon response that can drive and maintains the cancer stem cells^{50,51}.

Fas and FasL not only important as a cancer growth promoter but, unexpectedly, the elimination of Fas or FasL from cancer cells will induce the cancer cell to death ^{52,53}. Death induced by the Fas/FasL elimination (DICE) is a form of cell death that specifically affects cancer cells and cancer stem cells, which is not hampered by Bcl-xl expression and independent from p53, caspase-8, and RIPK1/MLKL pathway. Elimination of Fas/FasL is characterized by an increase of the production of reactive oxygen species (ROS) by mitochondria, aggregating the size of the cancer cell and DNA damage, which is similar to a necrotic form of mitotic catastrophe⁵³.

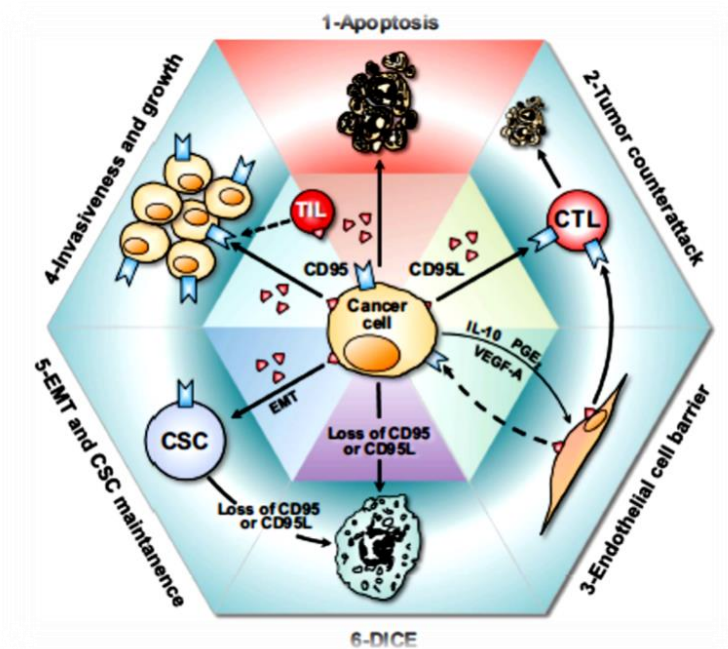


Figure 1.13. The role of Fas and FasL in cancer⁵⁴

1.2.5. FAS, TRANSCRIPTION FACTORS, AND APOPTOSIS

The tumor suppressor p53 is important in the regulation of apoptosis, cell cycle progression, and DNA repair. Activation of p53 depends on the response to various types of cellular stress like hypoxia, DNA damage, and oncogenic signaling. Mutation in TP53, which occurred in half of all human cancers, will affect the activity and stability of the p53 in regulating the apoptosis reaction. Nevertheless, the apoptotic response in tumors with wild type p53 might be hindered by the apoptotic cofactor's deficiencies⁵⁵.

In Fas-mediated apoptosis, Fas was significantly induced and is a critical regulator of p53-dependent apoptosis in response to hypoxia⁵⁶. P53 inactivation is involved in the reduction of apoptotic response mediated by Fas. A study done by Maecker et al. discovers that in oncogenic progression, tumor cells promoting their survival and evade immune surveillance by either to loss their p53 or malfunction the Fas sensitivity. Oncogenic activation and DNA damage are adequate stimuli to increase the p53-dependent transcription of Fas to create a selective condition in order to eradicate p53 or inactivate the components of the Fas death pathway⁵⁷.

Puma is a p53 downstream target and directly regulated by NF- κ B in human colon cancer cells⁵⁸. Crosstalk and defect in regulation between the Fas pathway and the NF- κ B pathway has a significant role in cellular fate. NF- κ B signaling is also stimulated upon Fas induction⁵⁹. NF- κ B may have an important role in promoting an apoptotic response through the up-regulation of c-Myc and p53 in the nucleus⁶⁰.

Apoptosis is not harmful to the host and does not induce any inflammatory reaction to compare with necrosis since the cell membrane is not ruptured and remains intact. The cells will break into apoptotic bodies, which will be rapidly engulfed by phagocytosis^{61,62}. TNF- α is important in inflammation, immunity, and cell survival regulation. However, the TNF- α axis has been discovered has diverse roles in promoting tumor progression and metastasis⁶³⁻⁶⁵. Apoptosis induction by Fas and FasL up-regulating the expression of TNF- α .⁶⁶

1.3. DEVELOPMENT OF OLIGONUCLEOTIDE NANOMEDICINE AGAINST HCC

In recent years, oligonucleotides nanomedicine represents a promising bench-to bedside strategy in medicine. In January 2017, after the evaluation of strictly controlled trials, the Food and Drug Administration approved the application of six oligonucleotides for therapy⁶⁷. This breakthrough is a very promising prospect for various oligonucleotides nanomedicine. Oligonucleotide nanomedicine has been demonstrated to be a powerful tool both for diagnostic and therapy. Modified oligonucleotide therapy has the potential to influence gene expression and improve the disease outcome without toxicity to normal cells^{68,69}. These nucleic acid-based technology has been widely studied, starting from anti-sense oligonucleotides, RNA interference (RNAi) to aptamer⁷⁰.

Anti-sense oligonucleotides (ASO) is a short strand nucleic acid that able to down-regulate target protein expression by hybridization with the complementary mRNA⁷¹. This hybridization will cause mRNA degradation, translational arrest, and mRNA maturation inhibition, which then convince a blockade in the transmission of genetic information from DNA to protein^{71,72}. The molecular mechanism responsible for ASO activities is divided into three phases: pre-hybridization, hybridization, and post-hybridization⁷³. Nowadays, modified ASO not only able to downregulate the gene expression but also can modulate the expression of the targeted gene⁷⁴.

The RNA interference (RNAi) is a revolutionary discovery in post-transcriptional gene regulation, which can act either as a gene silencer or inducer^{75,76}. RNAi has therapeutic potential in the future due to its specificity and robustness. RNAi consists of small interfering RNA (siRNA), short hairpin RNA (shRNA) and micro RNA (miRNA) which is a small noncoding regulatory RNA which first discovered and published in 1980⁷⁷. RNAi is a gene-silencing method with a specific sequence induced by double-stranded RNA (dsRNA). This approach is inexpensive and provides information about gene function quickly and easily. The use of RNAi for genetic-based therapies is widely studied in many diseases, especially cancers. RNAi-directed gene-silencing that combined with genomics data allows functional determination of any gene expressed in a cellular level or pathway level⁷⁸.

1.3.1. SMALL INTERFERING RNA (siRNA)

Small interfering RNAs (siRNAs), is one of the gene silencing methods that widely used to down-regulate gene expression. The process is guided by sequence complementarity and can be used therapeutically to block the synthesis of disease-causing proteins⁷⁹. siRNAs can be applied into cells as an exogenous dsRNAs or by nuclear transcription of miRNA genes⁸⁰. siRNA is an attractive new form of therapy that can be applied for cancer targeted therapy and other diseases. Moreover, siRNA can be used as a tool to study single gene function *in vitro* and *in vivo*⁸¹. siRNA is either burdened onto RISC directly or employ a Dicer mediated process upon entering the cytoplasm before commencing the RNA interference process via target mRNA cleavage and degradation mechanism (Figure 15) ⁸².

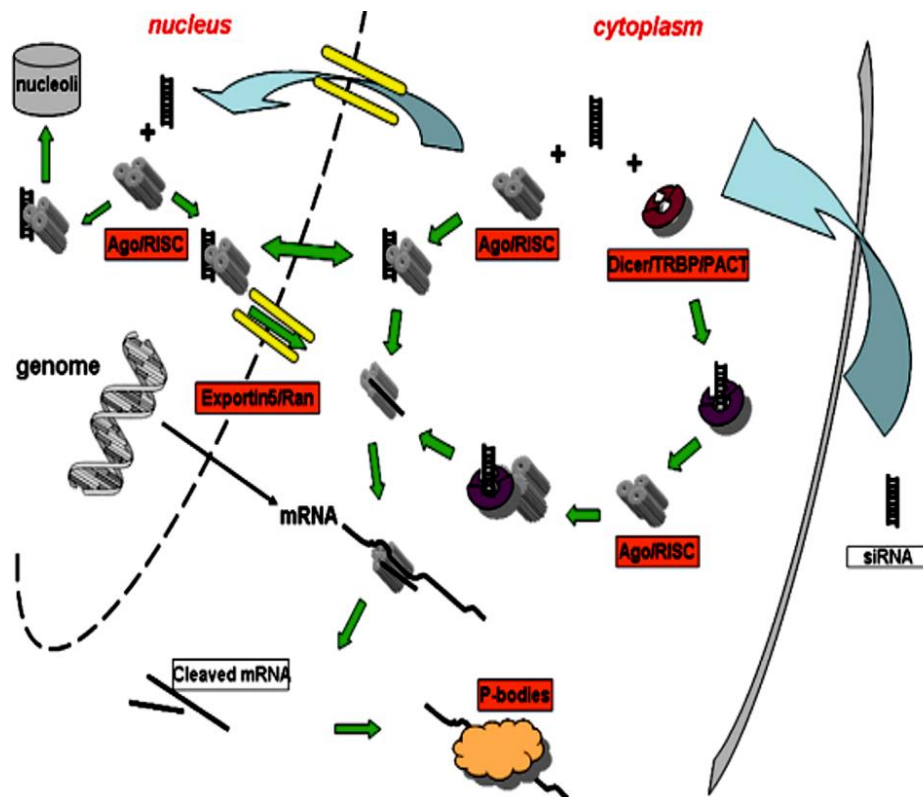


Figure 1.14. Schematic of the siRNA mediated RNA interference pathway⁸²

1.3.2. APTAMER

Aptamer is a single-stranded RNA or DNA oligonucleotides with low molecular weight (6-30 kDa) that specifically and efficiently bind to a target molecule^{70,83}. This characteristic makes them suitable for targeted therapy because of their ability to reach the core of the cancer cells and to internalize through the endosomal pathway. Aptamers have a flexible configuration that recognizes and binds to the related target in a specific and high binding affinity *via* an adaptive recognition manner⁸⁴. The aptamer-target complex has very low dissociation constants ranging from picomolar to nanomolar due to the specific hydrogen bonding⁸⁵⁻⁸⁷. The aptamers have a unique niche compared to other oligonucleotides. They can be developed to bind an intracellular or extracellular target, and they can be functioned as an agonist or antagonist (Figure 16)⁸⁸.

The principle of the aptamer molecular binding is based on its capability to spontaneously fold into a unique three-dimensional (3D) structure without the involvement of covalent bonds^{84,89}. As expected, the effect of aptamer-target interaction depends on the molecular function and cellular localization of the target molecule. In some cases, the aptamer-target complex can block the interaction between a ligand and its receptors that subsequently stimulates the cellular response⁹⁰ as for example, an immune response against viral infection^{91,92}. Some aptamers also have agonist-like activities that can enhance and induce protein synthesis^{93,94}.

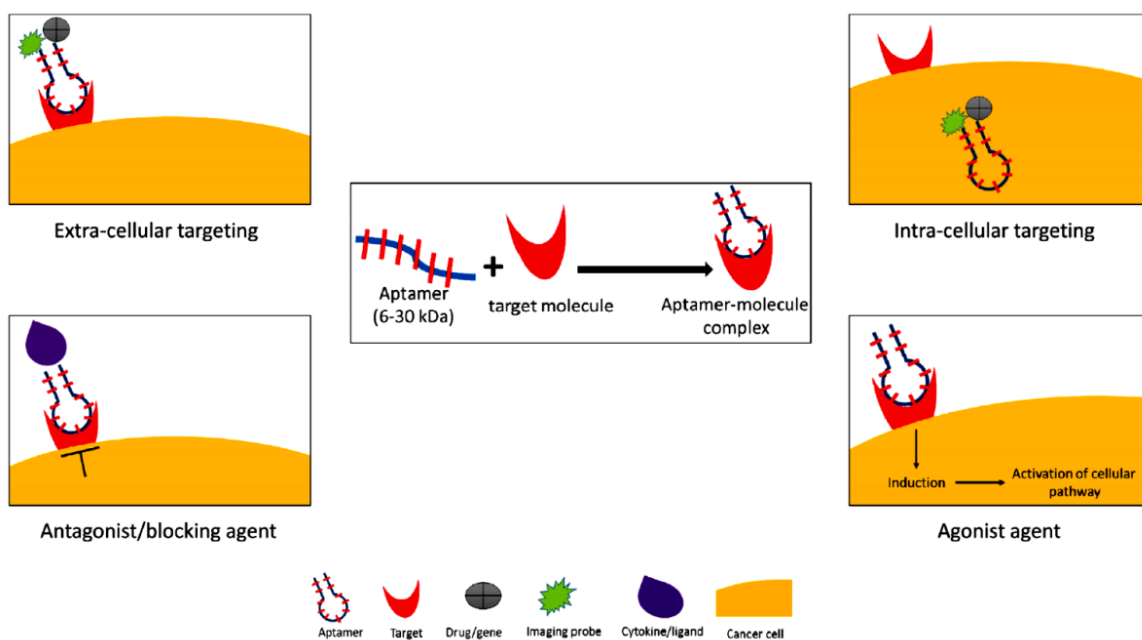


Figure 1.15. Applications and functions of aptamer⁸⁸

Aptamers are also known as "chemical antibodies" since they can bind specifically to target molecules either in the intracellular or extracellular environment^{85,95}. Aptamers have high versatility in targeting different molecules of different nature, size, and complexity, ranging from ions to whole-cell, antibiotic, protein, bacteria, and virus⁹⁶.

However, compared to conventional antibodies, aptamers exhibit significant advantages. Aptamer commonly sustains its specificity and sensitivity by binding its ligands *via* adaptive recognition involving conformational alteration and molecular shape complementary⁹⁷. They are stable at room temperature and in non-physiological conditions, non-immunogenic, non-toxic, and suitable for long-term repeated administration^{98–100}. They also have high bio-distribution in biological fluids^{101,102} and high capacity to penetrate and remain in the tumor site¹⁰³. The low-cost and well-standardized chemical synthesis, which is 1000 times cheaper than the antibodies production^{104,105}, are also important aspects.

The aptamer can be generated by using the Systematic Evolution of Ligands by Exponential Enrichment (SELEX), first described by Tuerk and Ellington^{106,107}. SELEX method

is based on a chemical process consisting of selection (binding phase, partitioning phase, and elution phase), amplification, and conditioning ¹⁰⁸. In the binding phase, a random nucleic acid library is incubated with the target molecule, followed by the partitioning phase that separates target-bound oligos from the remaining unbound library. The bound oligos are then eluted and are amplified to generate an enriched pool of aptamers candidates. The process can be repeated for 8-20 cycles to obtain candidates with the highest affinity to the target molecule. Finally, the sequences of chosen aptamers are characterized by sequencing ¹⁰⁹.

Since its launch in 1990, conventional SELEX has been progressively modified to improve aptamer specificity and affinity, to simplify the process and to increase time and cost efficiency ¹¹⁰⁻¹¹². The modifications in the SELEX method depend on the purpose and target molecules. For example, *in vivo* SELEX, cell-SELEX, one-round SELEX, *in silico* SELEX, capillary electrophoresis-SELEX, magnetic bead-based SELEX, and high-throughput sequencing-SELEX are several novel SELEX technologies that are already well established ¹¹³. The identification of aptamer against whole cells is called the Cell-Systematic Evolution of Ligands by Exponential Enrichment (SELEX). The process is consisting of several phases of selections that including of binding phase, partitioning phase, elution phase, amplification, and screening phase ¹¹⁴⁻¹¹⁶.

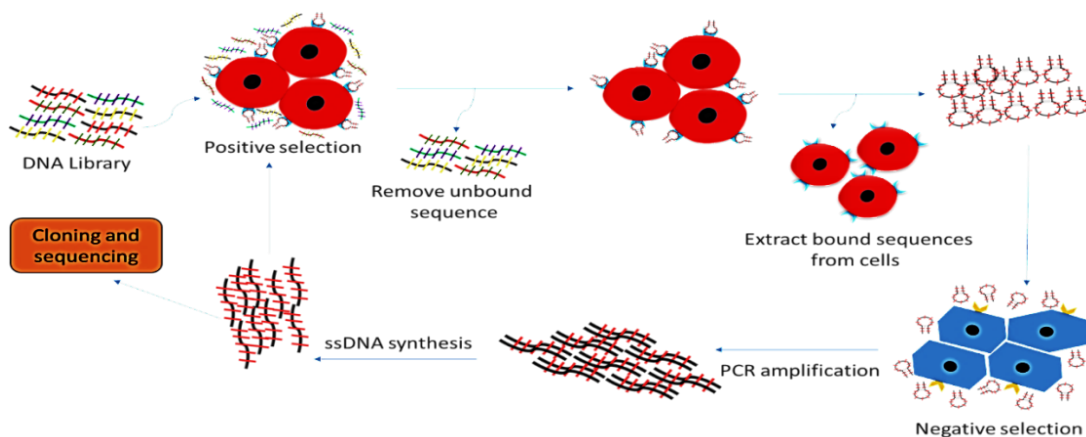


Figure 1.16. Aptamer selection using Cell-SELEX

In recent years, aptamer-based application is growing rapidly due to its promising function for diagnostic, targeted therapy, nano-imaging, and nano-delivery agent by conjugating the aptamer with an anti-cancer drug, nanoparticles, and gene therapy (Figure 18). Theranostic is a new medical approach with a combination of a diagnostic and therapeutic method. Theranostic is a promising method to overcome the limitation of conventional cancer therapy and diagnosis. Aptamer had considered as a powerful theranostic agent due to its versatility, which can be modified and improved to obtain a specific application to treat cancer. The theranostic aptamer was developed in many different ways due to its superior performance in solid tumor penetration over antibody. Aptamer can be functional both as a simultaneous diagnostic and therapeutic agent by combining its greater efficacy in targeting the cancer cells with its agonist/antagonist capability in cellular pathways⁸⁸.

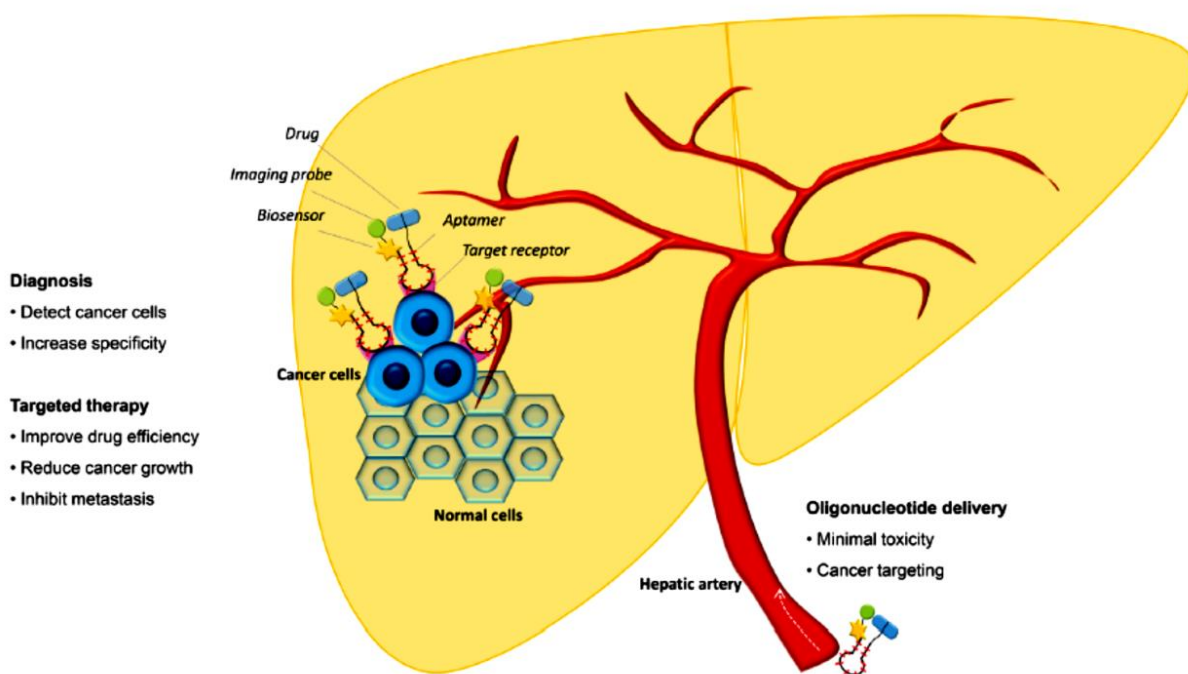


Figure 1.17. Aptamer for HCC diagnostic and targeted therapy⁸⁸

Chapter 2

RELEVANCE OF FAS DEATH RECEPTOR IN CLINICAL SAMPLES

*Parts of this study was presented at the 25th European Association of Cancer Research (EACR)
Congress in Amsterdam, 2018 and European Association for the Study of Liver (EASL)
Hepatocellular Carcinoma (HCC) Summit, Lisbon, 2019*

2. RELEVANCE OF FAS DEATH RECEPTOR AND FASL IN CLINICAL SAMPLES

2.1. BACKGROUND

The role of Fas and FasL related to cancer has been discovered in various types of human cancers tissues. Abberant expressions of Fas and FasL were observed in ovarian, cervical, endometrial carcinoma, colon tumor and gastric cancer's tissue samples form surgical resected patients¹¹⁷⁻¹¹⁹. Functional expression of Fas and FasL was also studied in human intraepithelial lymphocytes where the colon tumors can exploit the lymphocyte death program by FasL expression¹¹⁸. Anomalous expression of Fas and FasL are involved and responsible in carcinogenesis and metastasis of gastric cancer where the Fas expression level was significantly increased in normal tissue compare to carcinoma tissue samples. On the other hand, the FasL expression was significantly decreased in normal tissue compare to carcinoma tissue samples¹¹⁹.

Studies using clinical samples showed that Fas expression was significantly lower in hepatocellular carcinoma (HCC) tissue samples than in noncancerous tissues¹²⁰⁻¹²². In HCC, Fas was mostly expressed only in cytoplasm, whereas Fas expressed both in cytoplasm and on the surface of the noncancerous specimen¹²². Fas receptor might eliminated on the surface of hepatoma cells to escape from the host immune surveillance system and promote metastasis¹²¹. There was a correlation between the degree of differentiation and the Fas expression in HCC. There was a significant reduction of Fas expression in poorly differentiated HCC compare to well or moderately differentiated HCC¹²⁰.

The level of Fas and FasL expression can be used as a tool to predict the clinical stage and the future prognostic of the patients. Fas and FasL has a significant value for the measurement of the disease free survival (DSF) of HCC patients which can predict the recurrence of the disease¹²³.

Several *in vivo* studies showed that Fas resistance play a pivotal role in cancer defense and recurrences which can counterattack the immune system, promotes the tumor growth and increase the stemness of the cancer cells^{50,124}. Study of Fas in the ovarian cancer and liver cancer tissues suggesting that the absence of Fas hampered the cancer cells formation⁵³.

2.2. AIM

The main of this study is to focus on the importance of the Fas/FasL death receptor in HCC and its potential in developing future treatment of oligonucleotide nanomedicine. The main aim is divided into several sub-aims:

1. To show the relevance of Fas and FasL expression in HCC clinical samples
2. To study a correlation of Fas/FasL with other pro-apoptotic genes in HCC clinical samples
3. To observe the correlation of Fas and FasL with transcription factor in HCC clinical samples

2.3. MATERIAL AND METHODS

2.3.1. Clinical samples

Human liver tissues were collected from HCC patients undergoing liver resections and normal donor liver in the similar age group as control. Tissues were snap-frozen in liquid nitrogen or in RNAlater™ and stored in -80°C before further processing. The diagnosis of patients was established on international criteria together with its Edmondson Steiner HCC grading and other clinical findings. Informed consent to participate to the study was obtained from each patient or by a legal representative.

In the analysis, we used three different tissues from HCC patients (HCC, peri-HCC, cirrhosis) and normal liver tissue. Total tissue samples were 111 (39 HCC, 30 peri-HCC, 31 cirrhosis, and 11 normal). All the tissue samples were taken from patients that undergoing liver resection without any prior treatments.

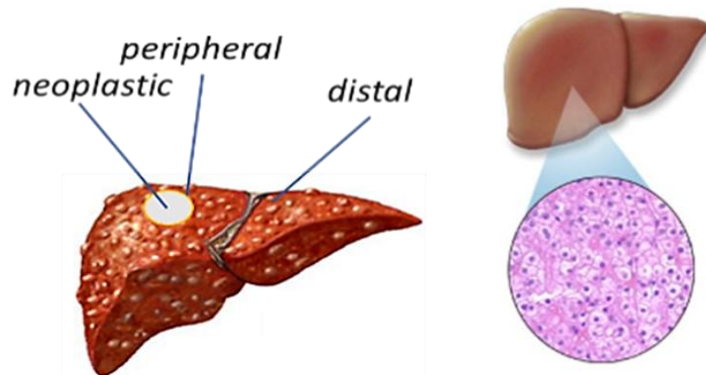


Fig. 2.1. HCC tissue and normal liver tissue

2.3.2. Protein expression analysis

Immunofluorescence

Immunofluorescence was performed using paraffinized HCC tissue samples. The samples were deparaffinized by heating the slides in the oven at 60°C for 45 minutes. The slides then deparaffinized using xylene for three times for 5 minutes. Several dilutions of ethanol were used to rehydrate the slides starting from 100%, 95%, 80%, 70% and 50%. The slides were washed for 1-2 times for 2 minutes before finally washed with distilled water. For antigen retrieval, the slides were heated in the microwave for 15 minutes with 10 mM Sodium citrate pH 6.0 + Tween20 0.05%.

The slides were then fixated with 4% PFA in PBS for 15 minutes before blocking with 5% fetal bovine serum (FBS) in PBS for 30 minutes. After tissues permeabilization with 0.5% Triton X100 in PBS, tissues were incubated overnight at 4°C with antibodies against human Fas (DX2, Miltenyi Biotech., Bergisch Gladbach, Germany) and human FasL (NOK-1, Santa Cruz Biotech., CA, USA) conjugated with fluorescein (dilution 1:100). Nucleus was stained with Hoechst 33258. Single immunostaining and IgG isotype control were also carried out. Proteins positivity was observed by using a fluorescence microscope Leica DM2000 (Leica Camera AG, Solms, Germany).

2.3.3. Gene expression analysis

Total RNA isolation and quantification

Total RNA extract from human liver tissues total RNA was obtained using TriReagent (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's protocol. The homogenized samples were incubated for 10 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. After the incubation, 200 µl of chloroform were added in the samples per 1 ml of Tri-Reagent. Samples were vortex vigorously for 15 seconds and incubate them at room temperature for 10 minutes before centrifuging the samples at 12,000 x g for 15 minutes in 4 °C. Following centrifugation, the mixtures were separated into lower red

phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The upper aqueous phase was transferred carefully into a new tube without disturbing the interphase. The volume of the aqueous phase was measured for the RNA precipitation (the volume of the aqueous phase is about 60% of the volume of TriReagent used for homogenization).

For the RNA precipitation process, 500 µl of isopropyl alcohol was added per 1 ml of TriReagent used for the initial homogenization. Then, the samples were incubated at 4°C for 10 minutes and centrifuged at 12,000 x g for 10 minutes at 4°C. To wash the RNA, the supernatant was completely removed and the RNA pellet was washed twice with 1 ml of 75% ethanol per 1 ml of TriReagent used for the initial homogenization. The samples were mixed and centrifuged at 7,500 x g for 5 minutes at 4°C. Finally, the RNA pellets were dried by air-dry for 5-15 minutes. The RNA was dissolved in DEPC-treated water by passing solution a few times through a pipette tip before kept in -80°C.

RNA concentration was quantified by measuring the absorbance at 260 nm in a DU730 spectrophotometer (Beckman Coulter, Fullertone, CA., USA). The RNA purity were evaluated under the MIQE guidelines by measuring the ratio A₂₆₀/A₂₈₀ with appropriate purity values between 1.8 and 2.0.

Reverse Transcription – quantitative Real Time PCR (RT – qPCR)

Reverse Transcription (RT) was performed to obtain cDNA from 1 µg of purified RNA using the High Capacity cDNA Reverse Transcription Kits (Applied Biosystem, USA) according to the manufacture's suggestions. The reaction was run in a thermal cycler (Bio-Rad) based on the protocol proposed by the manufacturer.

PCR amplification was carried out in 15 µl reaction volume containing 25 ng cDNA, 1 SYBR Green Supermix-composed by 100 nM KCl, 40 nM Tris-HCl, Ph 8.4, 0.4 nM each dNTP, 40 U/mL iTaq DNA polymerase, 6 mM MgCl₂, SYBR Green I, 20 mM fluorescein, and stabilizers (Bio-Rad), and 250 nM of gene specific forward and reverse primers. The reaction was run in CFX 9600 real time PCR system (Bio-Rad). The primer sequences were designed using

Beacon Designer 7.9 Software (PREMIER Biosoft International, Palo Alto, CA, USA) for the detection of the desired genes. The genes that were used in this study were listed in Table 2.1.

Table 2.1. List of primers used for Real Time PCR

Gene	Acc. no.	Forward primer (5' → 3')	Reverse primer (3' → 5')
18S*	NR_003286	TAACCCGTTGAACCCCAT	CCATCCAATCGGTAGTAGCG
β-actin*	NM_001101	CGCCGCCAGCTCACCATG	CACGATGGAGGGGAAGACGG
Fas/CD95	NM_000043.6	GCTGGGCATCTGGACCCTCCTACCT	CAGTCACTTGGGCATTAACACTT
FasL/CD95L	U11821.1	TGCCTTGGTAGGATTGGGC	GCTGGTAGACTCTCGGAGTTC
PUMA	NM_001127240	CCTGTAAGATACTGTATATGC	CCACTGTTCCAATCTGAT
p53	AB082923.1	CGGTGGGTTGGTAGTTTC	GATCCAGATCATCATATACAAGAG
CD90	NM_006288	CTCCAGCATTCTCAGCCACAAC	TCATCCTTACCTCCTTCTCCAACC

The reference genes were marked with asterisk (*).

2.3.4. Statistical analysis

Student's t-test was performed for statistical comparison between groups using software Instat version 3.05 (GraphPad Software, Inc., La Jolla, CA, USA). Data were obtained from at least three independent experiments and are expressed as mean ± SD. Statistical significance was set to p-value <0.05 and reported as indicated below: * P < 0.05, ** P < 0.01, and P < 0.001

2.4. RESULTS

2.4.1. Localization of Fas and FasL in human liver tissue

Immunofluorescence staining was performed using HCC tissue specimens to check the presence of Fas death receptor and FasL in liver cancer. The positivity of both Fas and FasL were observed in the HCC tissues (Figure 2.1).

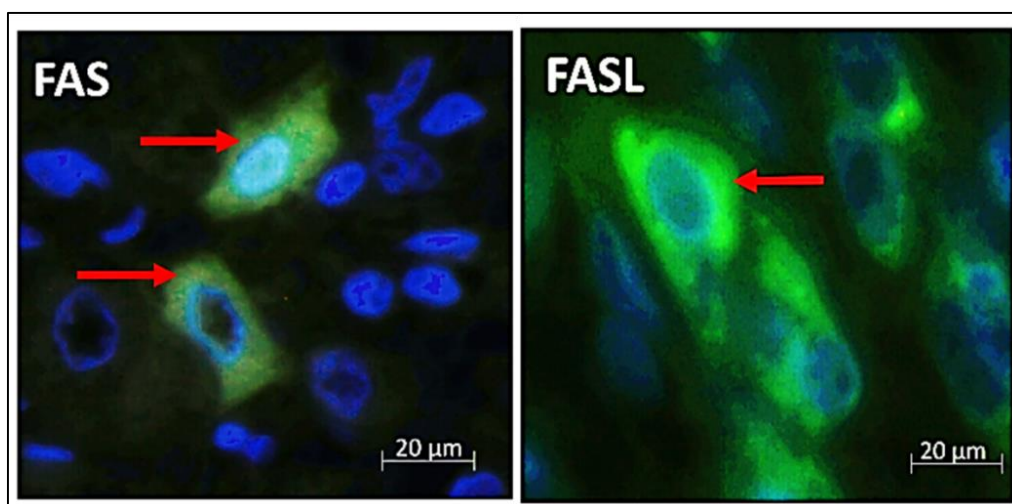


Figure 2.2. Fas and FasL immunostaining in human HCC tissue. Representative images of the Fas and FasL proteins in human HCC specimens. Green is the target protein, and blue is the nuclear staining.

2.4.2. Aberrant expression of CD95/Fas and CD95L/FasL in human HCC tissues

The mRNA expressions of Fas and FasL were analyzed in a cohort of human liver tissues consisted of normal donor liver, cirrhosis (distal), peri-HCC, and HCCs mentioned in Materials and Methods above. .

Fas death receptor and FasL were expressed in all hepatic tissues. Fas death receptor was significantly up-regulated in HCC compared to normal liver tissue. Regarding FasL, it was significantly up-regulated in cirrhosis, peri-HCC and HCC tissues compared to normal tissue samples as shown in Figure 2.2. (* $p < 0.05$, ** $p < 0.01$ vs normal).

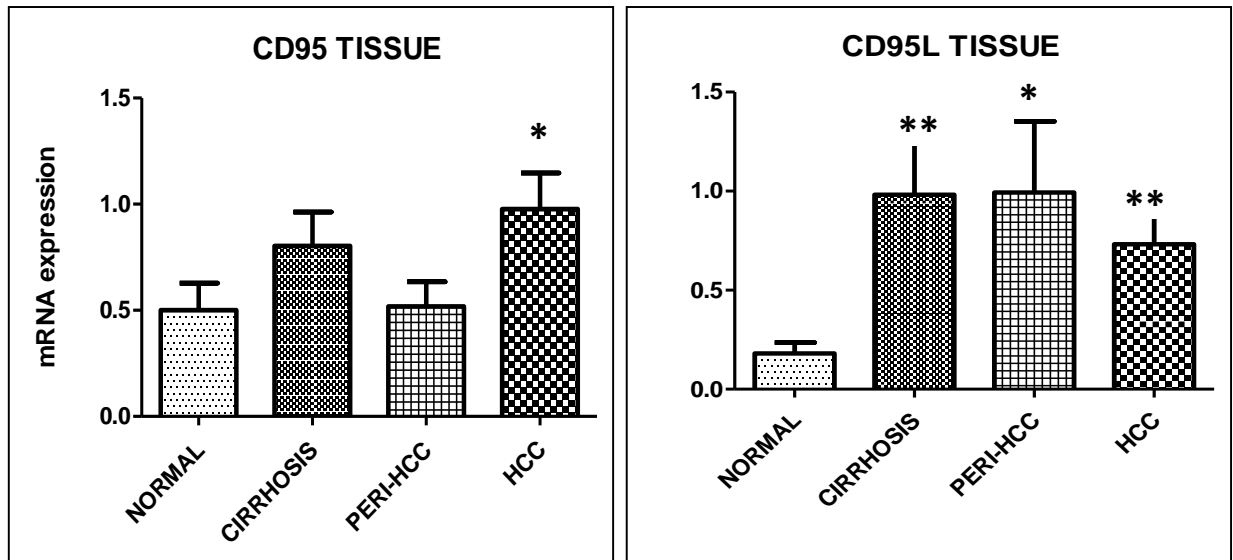


Figure 2.3. mRNA expression of CD95/Fas and CD95L/FasL in human HCC tissue. Fas death receptor was significantly up-regulated in HCC tissues compared with normal tissue samples. FasL was significantly up-regulated in cirrhosis, peri-HCC and HCC tissues compared with normal tissue samples (* $p < 0.05$, ** $p < 0.01$ vs. normal)

2.4.3. Fas and FasL expressions and patients clinical and pathological data

Since the expressions of Fas and FasL mRNA were significantly high in HCC as compared to normal tissues, their expressions in HCC nodules were further correlated with several clinical data of the patients, such as etiologies, gender, vascular invasion, and Edmonson-Steiner histological grading.

For the etiologies, we classified the HCC etiologies into 3 groups: Hepatitis B Virus (HBV)-related HCC, Hepatitis C Virus (HCV)-related HCC and others (metabolic/alcoholic-related HCC). Based on etiologies classification, even though the differences were not significant, Fas and FasL expressions were slightly high highly noticed in HCV-related HCC compared to HBV-related HCC and others (metabolic/alcoholic-related HCC) .

Genes expressions analysis were also demonstrated in HCC tissues based on the vascular invasion criteria since vascular invasion is important in cancer progression especially HCC.

Regarding on the expression of Fas death receptor and its ligand (FasL) in HCC based on vascular invasion, Fas was highly expressed in HCC tissues without vascular invasion compared to tissues with vascular invasion (Figure 2.4). The same result was also observed with FasL signature which also higher in the HCC tissues without vascular invasion compare to the HCC tissues with vascular invasion.

Fas and FasL were also assessed based on the Edmonson-Steiner grades to determine the correlation between their expressions and HCC histological grading. Regarding Fas death receptor, in Figure 2.5, we can see that there was no difference between grade G1/G2 and grade G3/G4. By the way, there was a difference expression of FasL between grade G1/G2 and G3/G4. We determined an up-regulation of FasL in grade G3/G4 compare to grade G1/G2.

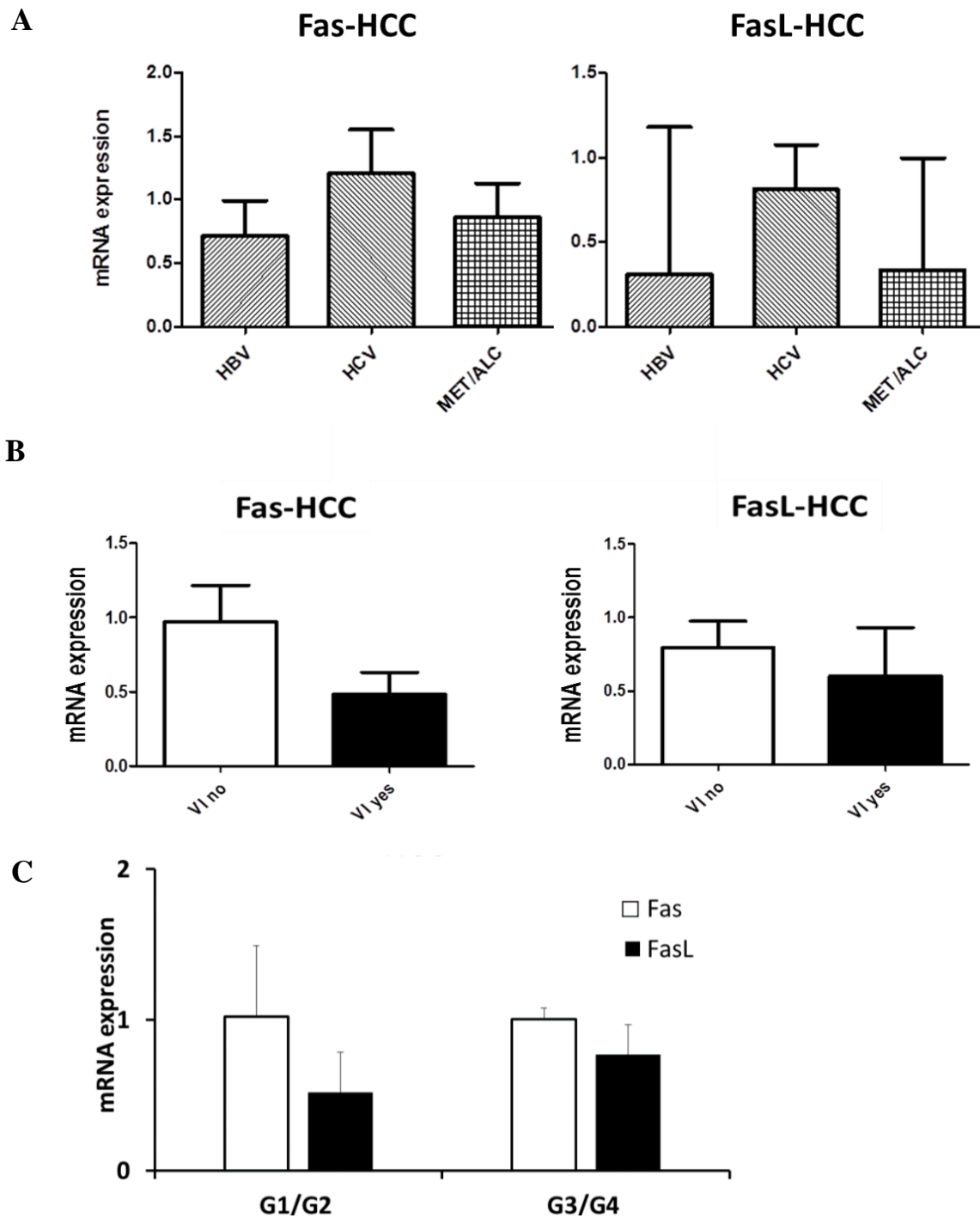


Figure 2.4. Expression of Fas and FasL based on several clinicopathological characteristics. A. Etiology. B. Vascular invasion in HCC tissues. C. Edmonson-Steiner histological grading.

2.4.4. p53 regulates the expression of Fas death receptor in HCC

Since p53 can regulate the expression of Fas death receptor, gene expression analysis was performed to assess the expression of transcription factor p53 in in the same set of cirrhosis and HCC tissue samples. As expected, our data showed that the p53 was increased in HCC tissue samples compared to cirrhosis tissue samples as shown in Figure 2.5 even though the difference was not statistically significant.

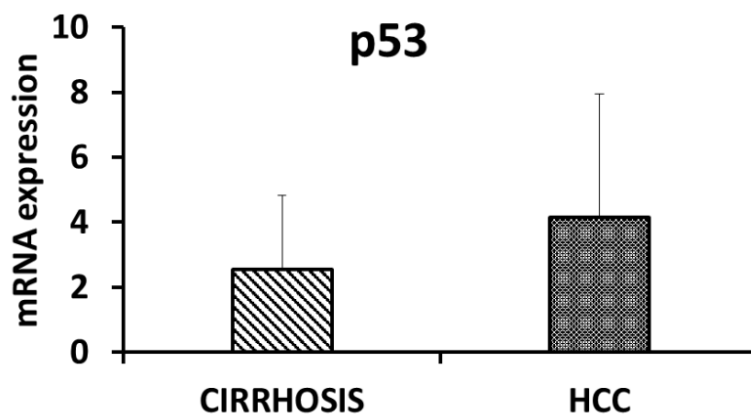


Figure 2.5. mRNA expression of p53 in paired cirrhosis and HCC tissue samples.

2.4.5. Deficiency of pro-apoptotic PUMA in HCC

Pro-apoptotic gene PUMA were assessed in the hepatic tissues: cirrhosis, peri-HCC and HCC in order to observe the relation between PUMA and HCC. PUMA gene was not expressed in all of the hepatic tissue (Table 2.2). PUMA expressed in 24 (77%) cirrhosis samples from total 31 cirrhosis samples. 70% of peri-HCC samples were expressed PUMA from total of 27 peri-HCC samples. Regarding HCC samples, PUMA was only detected in 13 (41%) HCC samples from total 32 HCC samples. The expression of PUMA decreased depends on the damaged of the liver (Figure 2.6).

Table 2.2. Percentage of samples that expressed PUMA in human hepatic tissues

Samples	N	PUMA percentage (%)
Cirrhosis	24/31	77%
Peri-HCC	19/27	70%
HCC	13/32	41%

(n=samples that expressed PUMA mRNA from total 90 samples)

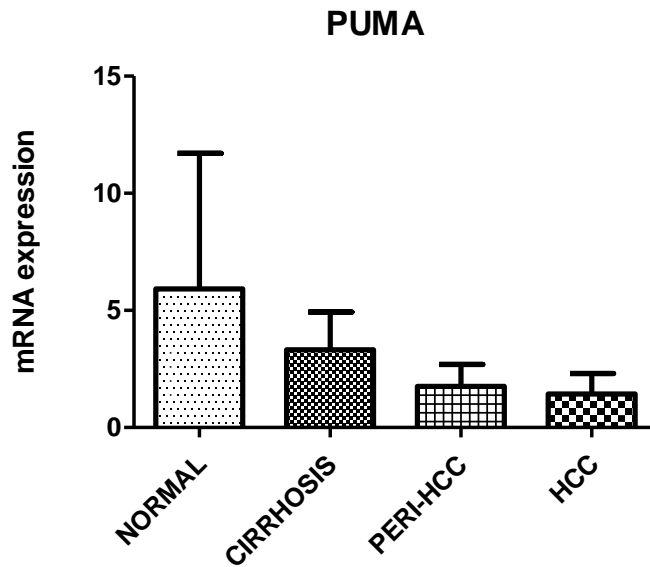


Figure 2.6. Expression of pro-apoptotic PUMA in human hepatic tissue.

2.5. DISCUSSION AND CONCLUSION

CD95/Fas death receptor is the key component of the extrinsic pathway that activates the apoptosis signal pathway by binding to its cognate ligand, Fas ligand (CD95L/FasL) that induces apoptosis of cancer cell⁴⁰. Dysfunction of Fas due to mutation will lead to inactivation of the Fas-mediated pathway resulting in survival and progression of the tumor cells⁴⁸. Moreover, chronic activation induced the cancer stemness^{50,51}.

The role of Fas and FasL related to cancer has been studied in various types of human cancers tissues. However, its relevance in HCC, especially for European samples is still needed to be defined. In this study, the mRNA expressions of Fas and FasL were evaluated in HCC clinical samples compared to its paired peri-HCC and cirrhotic (distal) tissues, together with a panel of healthy donor livers as control.

The expressions of Fas and FasL mRNA were highly variable in human liver tissues. However, both Fas and FasL expression levels were found to be high and they were significantly up-regulated in HCC compared to normal tissues. Interestingly, FasL was significantly increased,

already started from liver cirrhosis to HCC, indicating the relevance of this gene in the progressive damages of the liver. We hypothesize that it might be one mechanism by which damaged cells would escape Fas-mediated pathway.

Regarding the correlation between Fas/FasL expressions with clinical and pathological data of the patients, we checked the level of mRNA expressions of the Fas/FasL with etiologies, vascular invasion, and histology. We observed that higher Fas (and slightly FasL) expressions was noticed HCV-related HCC. It might be related to the importance and the role of Fas death receptor and Fas ligand in immune system homeostasis¹²⁵.

Recurrence with poorer survival is associated with vascular invasion on histopathology. However, in this study, the expression of Fas and FasL were higher in HCC nodules without any vascular invasions. This finding suggested that high expression of Fas and FasL were not correlated with cancer recurrence and poorer survival. Regarding histology, we noticed that FasL expression was increased in histologically poor differentiated HCC, graded as Edmonson-Steiner grade 3 and 4 (ES G3/G4) compared to well and moderate differentiated HCC ES G1/G2.

TP53 regulates the expression of Fas death receptor and cell's sensitivity to apoptosis response by allowing cytoplasmic Fas death receptors redistribute to the cell surface¹²⁶. Our findings might suggest there would be an involvement of transcription factor p53 in regulating the Fas death receptor expression in HCC. Moreover, the loss of apoptotic function of Fas death receptor might be linked to p53 alterations that contribute to the self-maintenance and survival of cancer cells¹²⁷. Alteration of p53 gene mainly related to mutation that occurred in half of all human cancers. It might affect the activity and stability of the p53 in regulating apoptosis. In oncogenic progression, tumor cells promote their survival and evade immune surveillance by either to loss their p53 or malfunction the Fas sensitivity⁵⁷.

Fas mutation is not the only cause of tumor growth and progression in the Fas-mediated apoptosis pathway⁴⁸. Tumor progression is also supported by other regulating factors involved in the apoptosis pathway. Based on this study, the absence and down-regulation of pro-apoptotic PUMA in HCC tissues indicated its important loss in normal apoptosis pathway in HCC. As known, PUMA is activated by the cell death stimuli. Deficiency of PUMA may decrease rate of

normal cell death. It implied an increased risk for cancer cells progression and therapeutic resistance^{128,129}.

In conclusion, aberrant expression of Fas and FasL were noticed in HCC tissues. They involved in pathogenesis and progression of HCC. We also showed that the expression of Fas was possibly regulated by the transcription factors p53, thus suggesting the involvement of p53 in Fas-mediated apoptosis pathway in HCC tissues. It will affect to the dysfunction of Fas in mediating apoptosis¹²⁷. Moreover, the deficiency of apoptotic gene PUMA in HCC may promote the cancer survival.

Dysregulation of Fas was related with HCC development, where HCC might have resistant mechanisms against Fas-mediated apoptosis that cross-linked with p53 and PUMA. Further study is need to evaluate the importance of Fas death receptor in HCC and the other related factors that involved in the dysfunction of Fas-mediated apoptosis.

Chapter 3

***IN VITRO* MODEL TO STUDY THE EXPOSURE OF FAS DEATH RECEPTOR**

*Parts of this study was presented at the European Association of Cancer Research (EACR)
Congress in Amsterdam, 2018 and European Association for the Study of Liver (EASL)
Hepatocellular Carcinoma (HCC) Summit, Lisbon, 2019*

3. *IN VITRO* MODEL TO STUDY THE EXPOSURE OF FAS DEATH RECEPTOR

3.1. BACKGROUND

Expression of Fas and FasL have been studied in various types of human cancer cell lines including breast cancer, colon cancer, gastrointestinal cancer, hepatocellular carcinoma, lung cancer, melanoma, ovarian cancer, pancreatic cancer and thyroid cancer⁵⁴. In multiple apoptosis-resistant tumor cells study, induction of Fas death receptor by FasL increased the motility and invasiveness of the cancers by NF- κ B and mitogen-activated protein (MAP) kinase pathways activation and by the involvement of urokinase plasminogen activator (uPA) which is one of the best-established marker proteins of tumor motility and invasiveness. The data also determined that FasL has tumorigenic activities could become extremely important during chemotherapy¹³⁰.

In another study, triggering Fas increased the metastatic ability and activation of epithelial–mesenchymal transition (EMT) in a colon cancer cell line resistant to oxaliplatin. Moreover, continuous exposure to oxaliplatin triggered Fas and stimulated tumor promoting pathways and cell migration. Fas can shift its role during the acquisition of chemo-resistance. As performed in this study, chemotherapy was performed as a selective method for resistant clones. Those resistant clones displayed a mesenchymal phenotype and expressed Fas with non-apoptotic function¹³¹.

Fas/FasL axis was also related with cancer stemness. Stimulation of Fas increased the cancer stemness in breast cancer, ovarian, renal, colon cancer, and glioblastoma cell lines⁵¹. In breast cancer cell lines, Fas exposure increased the prevalences of cancer stemness traits (e.g. phenotype) through TNF type I and STAT1 activation⁵⁰. The important of Fas related to the tumor growth and invasiveness were also demonstrated by knockdown of either Fas or FasL. The knockdown process lead to the induction of cell death in 12 cancer cell lines representing cervical, ovarian, liver, breast, renal, colon cancer, neuroblastoma or glioblastoma¹²⁴ and reduced the tumor growth of ovarian, liver, colon, and breast cancer cell lines⁵³.

3.2. AIMS

The main aim of this chapter is to reveal the importance of the Fas death receptor in various HCC cell lines by inducing and inhibiting Fas expression and its potential in developing the oligonucleotide nanomedicine. The main aim is divided into 2 sub-aims:

1. To discover the effect of Fas stimulation in HCC cell lines
2. To study the correlation of Fas with apoptotic genes, transcription factors, oncogene, cancer stem cells, and inflammatory genes that are important in apoptosis pathway and tumor development

3.3. MATERIAL AND METHODS

3.3.1. Cell lines

HCC human cell lines HepG2, JHH6, and Huh7 were used in representing from high to low basal expressions of Fas/CD95 and FasL/CD95L. Immortalized hepatocyte, IHH cell line, was used as normal cell control.

IHH, Huh7, HepG2 and JHH6 cells were cultured under sterile conditions and in their respective growth media until 90%-100% confluence. IHH was cultured in Dulbecco's Minimum Essential Medium-F12 1:1 (DMEM-F12) medium, supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA; USA) and 1% IU/mL penicillin-streptomycin, 1% L-glutamine, dexamethasone and insulin. HepG2 and Huh7 (well-differentiated HCC) were cultured in Dulbecco's Minimum Essential Medium (DMEM) with high glucose supplemented with 10% FBS and 1% IU/mL penicillin-streptomycin and 1% L-glutamine. JHH6 (poor-differentiated HCC) was cultured in Williams E medium supplemented with 10% FBS and 1% IU/mL penicillin-streptomycin and 1% L-glutamine. Cells were maintained in standard conditions, at 37°C in a humidified atmosphere with 5% CO₂. All cell lines were obtained from the Italian Liver Foundation cells collection.

3.3.2. Protein analysis

Flow cytometry

The presence of Fas and FasL protein was detected using human anti-Fas (DX2, Miltneyi Biotec GmbH, Germany), anti-FasL (NOK-1, Santa Cruz). IgG antimouse-FITC antibody (Jackson ImmunoRes.) was used as secondary antibody, when necessary. All cells were cultured until 80-90% confluence under optimal condition. The cells were washed two times with phosphate buffer saline (PBS) prior to detachment. Trypsin was used to detach the monolayer cells and neutralized with PBS containing 10% FBS. After detachment, cell suspensions were washed several times with cold wash buffer (PBS + 0.5% BSA + 3mM EDTA) and centrifuge at 1300 rpm for 5 minutes in 4°C.

The cells were filtered with 40 um cell strainer to obtain single cell suspensions. At least 5-6 million cells/ml were incubated with antibody for 60 minutes on ice in the dark. The cells were washed twice and then centrifuged at 1300 rpm for 5 minutes in 4°C to remove the unbound antibody. Flow cytometry analysis was performed immediately using a FACS Calibur flow cytometer (Becton Dickinson, New Jersey, USA). Ten thousands events were analysed per samples. Data were presented as a mean for at least three independent experiments.

3.3.3. Induction of extrinsic apoptosis pathway by Fas antibody

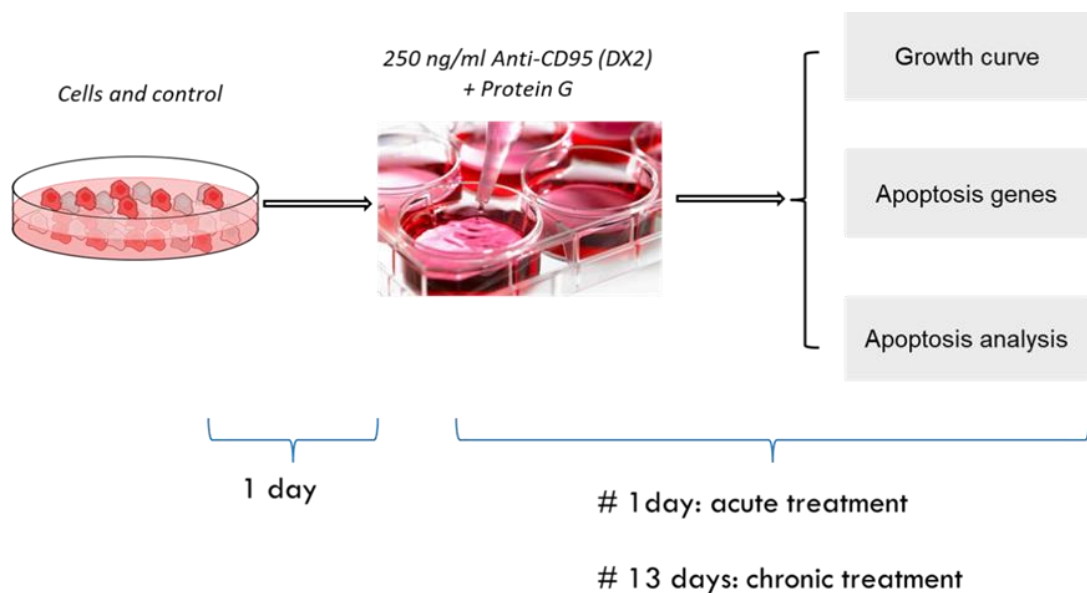


Figure 3.1. Workflow for apoptosis induction by anti-Fas

Growth curve assay

Growth curve assays were performed by treated and checked the effect of the treatment on HepG2 growth. The treatment were performed by using anti-Fas (clone: DX2) at a concentration of 250 ng/ml for 24 hours (acute treatment) until 13 days (chronic treatment) to induce apoptosis.

HepG2 cells were plated at the concentration of 10,000 cells/cm². After 24 hours, growth medium was changed and anti-Fas antibody was added in a fresh medium. Growth medium was changed on the fourth and the seventh days. Cells amount were counted every day by using a Bürker chamber under a light microscope (Leica). The data represented the mean \pm SD of three independent experiments. Total RNA and protein extract were kept for further analysis. Methods are described later in the chapter.

Apoptosis assay

Apoptosis assay was performed to study the early and late apoptosis after the anti-Fas (DX-2) induction in all cell lines studied (IHH, Huh7, HepG2, and JHH6). The analysis was performed by using Annexin V/PI staining kit (Apotest-FITC, VPS Diagnostics, Netherlands) based on manufacturer's protocol. Cells were plated at the initial concentration of 10,000 cells/cm² in 25 cm² flask. After 24 hours, apoptosis-induction was performed by adding anti-FAS (DX2) at a concentration of 250 ng/ml. Flow cytometric analysis was performed on day 1, 5, 7 and 13 after plating.

In brief, at each time point indicated, cells were washed with 5 ml of cold PBS and detached with 1 ml trypsin. After removing the trypsin, cells were suspended with concentration of 5×10^5 in binding buffer. Cells were centrifuged at 1200 rpm for 10 minutes at 4°C, then they were resuspended with 320 μ l of binding buffer (manufacturer's suggestion) and mixed. A mix of Annexin-V/PI (0.3 μ l Annexin V + 10 μ l of binding buffer + 9 μ l of PI) was added into each sample followed by cold incubation for 10 minutes. Flow cytometry analysis was performed immediately using a FACS Calibur flow cytometer (Becton Dickinson, New Jersey, USA). Ten thousands events were analyzed per samples. Data were presented as a mean for at least two independent experiments.

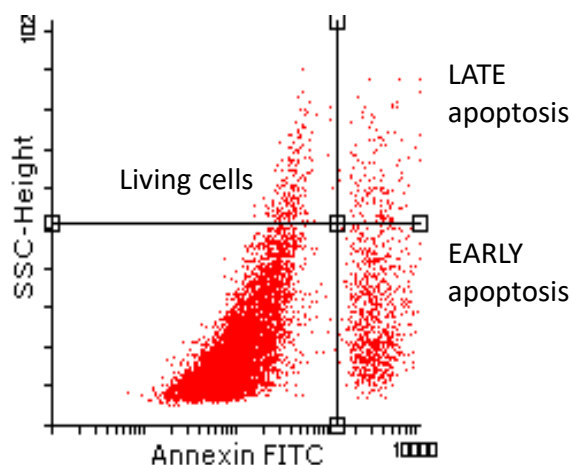


Figure 3.2. The determination of apoptosis signature for the induction study by anti-Fas

3.3.3. Gene expression analysis

Total RNA isolation and quantification

Total RNA from all cell lines were obtained using TriReagent (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's protocol. Homogenized samples were incubated for 10 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. After the incubation, 200 μ l of chloroform were added in the samples per 1 ml of TriReagent. Samples were vortex vigorously for 15 seconds and incubate them at room temperature for 10 minutes before centrifuging the samples at 12,000 x g for 15 minutes in 4 °C. Following centrifugation, the mixtures were separated into lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The upper aqueous phase was transferred carefully into a new tube without disturbing the interphase. The volume of the aqueous phase was measured for the RNA precipitation (the volume of the aqueous phase is about 60% of the volume of TriReagent used for homogenization).

For the RNA precipitation process, 500 µl of isopropyl alcohol was added per 1 ml of TriReagent used for the initial homogenization. Then, the samples were incubated at 4°C for 10 minutes and centrifuged at 12,000 x g for 10 minutes at 4°C. To wash the RNA, the supernatant was completely removed and the RNA pellet was washed twice with 1 ml of 75% ethanol per 1 ml of TriReagent used for the initial homogenization. The samples were mixed and centrifuged at 7,500 x g for 5 minutes at 4°C. Finally, the RNA pellets were dried by air-dry for 5-15 minutes. The RNA was dissolved in DEPC-treated water by passing solution a few times through a pipette tip before kept in -80°C.

RNA concentration was quantified by measuring the absorbance at 260 nm in a DU730 spectrophotometer (Beckman Coulter, Fullertone, CA., USA). The RNA purity were evaluated under the MIQE guidelines by measuring the ratio A₂₆₀/A₂₈₀ with appropriate purity values between 1.8 and 2.0.

Reverse Transcription – quantitative real time PCR (RT – qPCR)

Reverse Transcription (RT) was performed to obtain cDNA from 1 µg of purified RNA using the High Capacity cDNA Reverse Transcription Kits (Applied Biosystem, USA) according to the manufacture's suggestions. The reaction was run in a thermal cycler (Bio-Rad) based on the protocol proposed by the manufacturer.

Real time PCR was performed according to the SYBR Green Supermix protocol (Bio-Rad Laboratories, Hercules, CA, USA). PCR amplification was carried out in 15 µl reaction volume containing 25 ng cDNA, 1 SYBR Green Supermix-composed by 100 nM KCl, 40 mM Tris-HCl, pH 8.4, 0.4 mM each dNTP, 40 U/mL iTaq DNA polymerase, 6 mM MgCl₂, SYBR Green I, 20 mM fluorescein, and stabilizers (Bio-Rad), and 250 nM of gene specific forward and reverse primers. The reaction was run in CFX 9600 real time PCR system (Bio-Rad). The primer sequences were designed using Beacon Designer 7.9 Software (PREMIER Biosoft International, Palo Alto, CA, USA) for the detection of the desired genes. The genes that were used in this study were listed in Table 3.1.

Table 3.1. List of primers used for Real Time PCR

Gene	Acc. no.	Forward primer (5' → 3')	Reverse primer (3' → 5')
18S*	NR_003286	TAACCCGTTGAACCCATT	CCATCCAATCGGTAGTAGCG
β-actin*	NM_001101	CGCCGCCAGCTCACCATG	CACGATGGAGGGGAAGACGG
Fas/CD95	NM_000043.6	GCTGGGCATCTGGACCCTCTACCT	CAGTCACTTGGGCATTAACAATT
FasL/CD95L	U11821.1	TGCCTTGGTAGGATTGGGC	GCTGGTAGACTCTCGGAGTTC
PUMA	NM_001127240	CCTGTAAGATACTGTATATGC	CCACTGTTCCAATCTGAT
NOXA	NM_021127.2	AGTGTGCTACTCAACTCA	GTCCTGAGCAGAAGAGT
TNF-α	NM_000594	GTGAGGAGGACGAACATC	GAGCCAGAAGAGGTTGAG
NF-κB	L1906.1	GAATGCTGTGCGGCTCTG	CACGATTGTCAAAGATGGGATG
TERT	NM_198253	GTCATCGCCAGCATCATC	TGGAGGTCTGTCAAGGTAG
BIM	AY352518.1	ATCCCCGCTTTTCATCTT	GTGTCAAAGAGAAATACCCA
BAX	NM_001291428.1	TCGCCCTTTTCTACTTTG	CCCATGATGGTTCTGATC
p53	AB082923.1	CGGTGGGTTGGTAGTTTC	GATCCAGATCATCATATACAAGAG
MYC	NM_002467.4	AAACACAACTTGAACAGCTAC	ATTTGAGGCAGTTTACATTATGG
CD24	NM_013230.3	TGAAGAACATGTGAGAGGTTTGAC	GAAAACTGAATCTCCATTCCACAA
CD13	NM_001150	CCACCTTGGACCAAAGTAAAGC	TCTCAGCGTCACCTGGTAGGA
HIF1-α	NM_001530	CAGCAGTTACTCATGGAATA	AACCATACAGCATTTAAGAATC
CD44	NM_000610.3	CTCATACCAGCCATCCAATG	GAGTCCATATCCATCCTTCTTC
CD133	NM_006017	CATCTGCTCTCTGCTGAC	AACTTAATCCAACCTCAACC
CD90	NM_006288	CTCCAGCATTCTCAGCCACAAC	TCATCCTTACCTCCTTCTCCAACC
TNFR1	NM_001065	ATCCCTCCTTTCATTGG	GGCTTAGTAGTAGTTCCTTC

The reference genes were marked with asterisk (*).

3.3.5. Fas silencing by siRNA

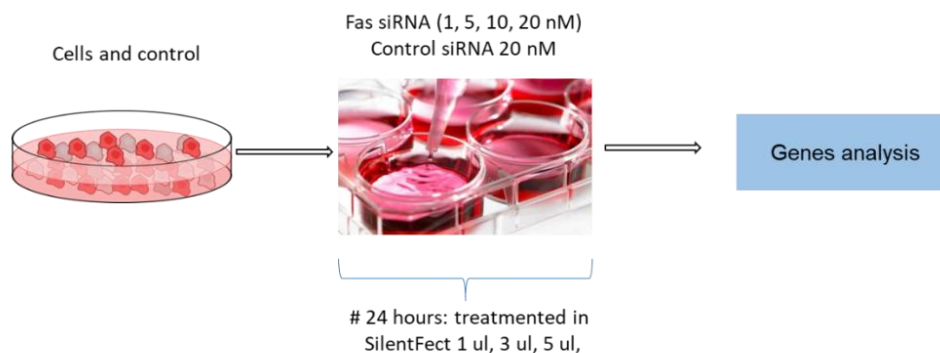


Figure 3.3. Workflow for Fas gene silencing by siRNA

The result of this part was described in the thesis of Alessia Di Nella for Master's Degree in the Course of Medical and Pharmaceutical Biotechnologies, University of Trieste, 2019.

For this study, Fas expressions and functions in hepatic cell lines were blocked by using Fas siRNA (SC29311, Santa Cruz). SilentFECT (Bio-Rad) was used as the transfection agent and scrambled siRNA (SC-37007, Santa Cruz) was used as a control siRNA in each experiment. After culturing, the cells were confirmed to be in optimal condition and confluence before used. The cultured cells were detached by using Trypsin. The single cells suspension were counted using Bürker chamber.

The cells were plate in two 6-well plates with total of 450,000 cells (concentration: 225,000 cells/ mL). Transfections were performed after the cells reached 60-80% confluence. Completed medium (10% Fetal Bovine Serum (FBS) (GIBCO) and 1% L-glutamine) without antibiotic were used for the cell's preparation. The transfection medium only contained basal medium and 1% L-glutamine without antibiotic and FBS. The protocol was based on the siRNA protocol with several modifications. The stock of siRNA (10 μ M) was diluted with RNase-free water at the working concentration (2 μ M). The appropriate amount of siRNA was diluted in 100 μ l of

transfection medium (based in the Table 3.2). The appropriate amount of SilentFect Lipid reagent was added into the mixture. The mixture was incubated for 45 min in room temperature to allow the formation of transfection complexes. The medium of the cells were changed with 1 ml transfection medium before adding the transfection complexes. The plates were gently swirled to ensure uniform distribution of the transfection complexes. The cells were incubated under their normal growth conditions for 8 hours. After the incubation, 1 ml of completed medium was added into the cells and incubated for another 24 hours. After 24 hours silencing with siRNA, the medium was aspirated and 500 ul of Tri-reagent was added for further experiment.

Table 3.2. Experimental set up for Fas silencing optimization

	Group 1			Group 2			Group 3		
SilentFect	1 ul			3 ul			5 ul		
siRNA (nM)	0	1	5	0	1	5	0	1	5
	10	20	mock	10	20	mock	10	20	mock

3.3.6 Statistical analysis

Student's t-test was performed for statistical comparison between groups using software Instat version 3.05 (GraphPad Software, Inc., La Jolla, CA, USA). Data were obtained from at least three independent experiments and are expressed as mean \pm SD. Statistical significance was set to p-value <0.05 and reported as indicated below:

* P < 0.05, ** P < 0.01, and P < 0.001

3.4. RESULTS

3.4.1. Expression of Fas and FasL in human hepatic cell lines

In our previous study, both Fas and FasL were expressed and up-regulated in the HCC tissue samples. We performed several experiments using an *in vitro* model to support our earlier results. The screening of the Fas and FasL positivity were performed in several human hepatic cell lines with a different morphological degree of differentiation: IHH (immortalized hepatocytes), HepG2 and Huh7 (well-differentiated HCC), and JHH6 (poor-differentiated HCC), as shown in Figure 3.1.

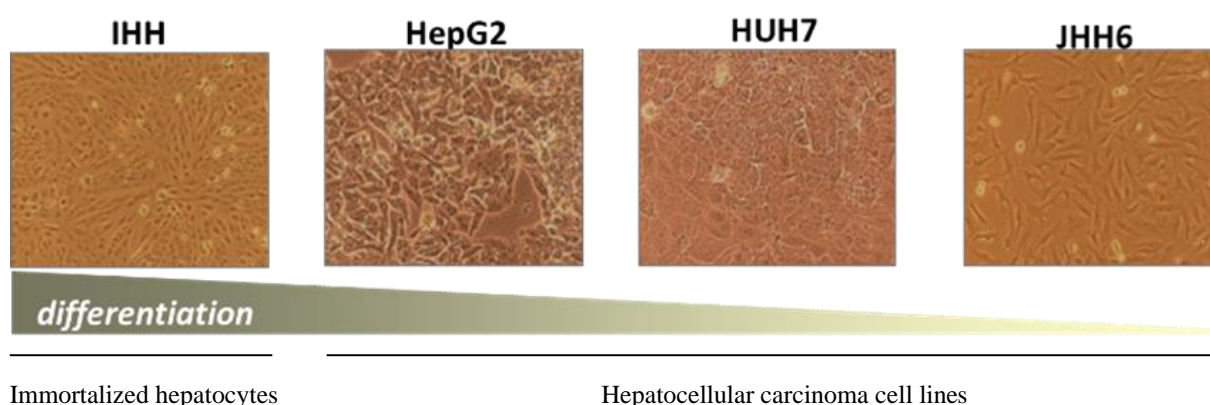


Figure 3.4. Human hepatic cell lines used in the study. Morphological differentiation of human HCC and non-HCC cell lines: IHH cell is immortalized hepatocytes, HepG2, and Huh7 cell lines are categorized in well-differentiated HCC and JHH6 cell line is categorized in poor-differentiated HCC.

Flow cytometer analyses were performed using antibodies that recognized Fas and FasL to detect the expression of the Fas and FasL in human hepatic cell lines. Around 50-60% of Fas protein was detected in the IHH cell line, and around 70-90% of Fas protein was detected in the HepG2 cell line. In contrast, in Huh7 and JHH6 cell lines only expressed around 1- 5% of Fas protein. The FasL protein was only expressed less than 2% in all of the hepatic cell lines. The mRNA data of Fas and FasL in hepatic cell lines supported the flow cytometer analysis (data not shown).

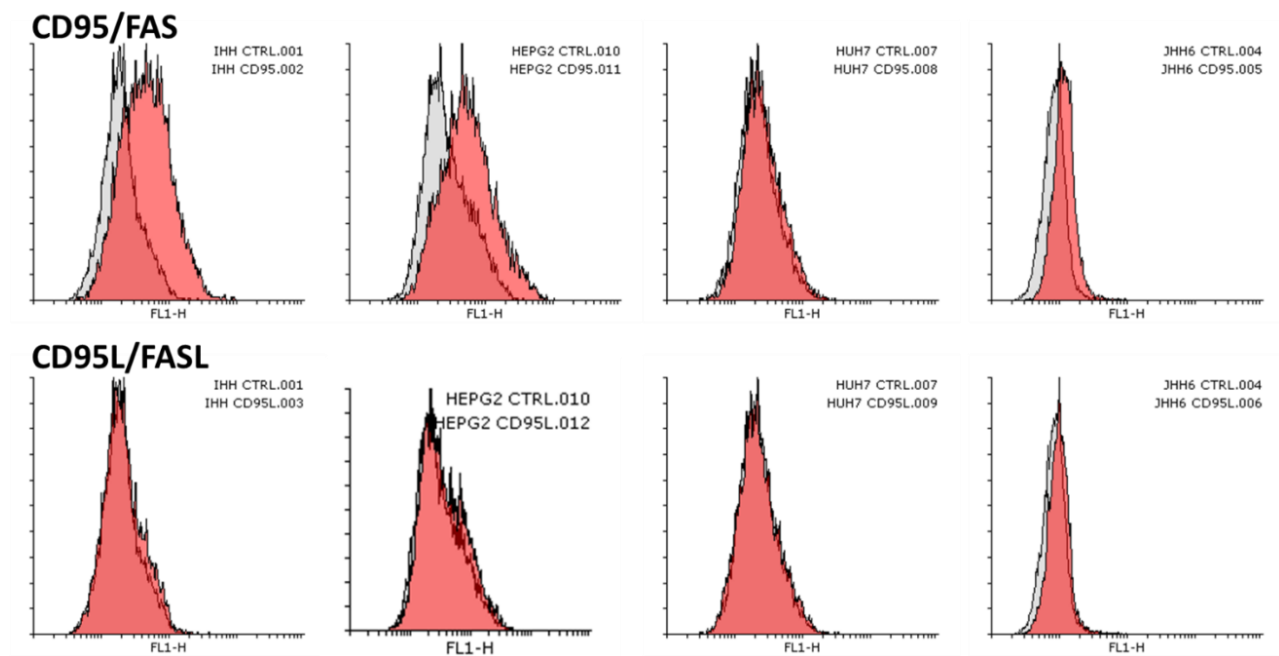


Figure 3.5. Basal expression of Fas and FasL in hepatic cell lines measured by flow cytometry. The upper panel and lower panel represent the fluorescence obtained by labeling with antibody anti-Fas or anti-FasL, respectively.

Based on flow cytometry and mRNA data, subsequent works (e.g. growth curve, silencing, and aptamer development) were conducted mainly on Fas experiments on IHH and HepG2 cells.

3.4.2. Chronic Fas death receptor induction promotes tumor growth

To evaluate the short (acute – 1 day) and long (chronic – up to 13 days) functional activity of Fas exposure (antibody anti-Fas DX2) to cells growth, growth curve analysis was performed to determine the non-apoptotic function of the Fas in HepG2, with anti-Fas DX2 250 ng/ml (+FAS) compared to control (CTRL). In this experiment, HepG2 cells growth was determined by cell counting from day 1 until day 13.

As seen from Figure 3.6., DX2 exposure significantly increased the growth of HepG2 cells only after chronic Fas induction from 10 days after plating compared to CTRL (t-test:** p<0.01 vs CTRL). It seemed that anti-Fas would need a sufficient time to give effect to the growth of cancer cells.

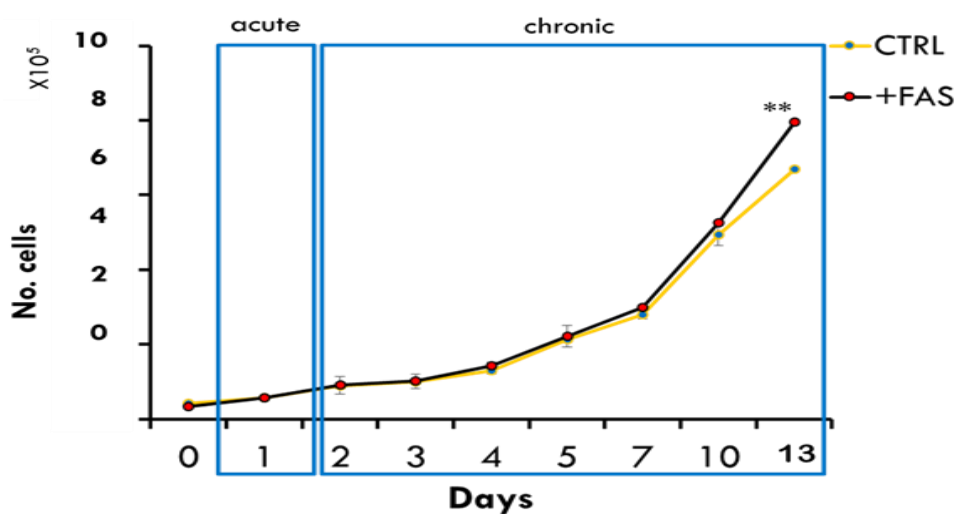


Figure 3.6. Growth curve of HepG2 cell after chronic induction of Fas. The growth of HepG2 cell was significantly increased after chronic induction of FAS (DX2; +FAS) compare with control (CTRL) (t-test:** p<0.01 vs. vontrol)

3.4.3. Chronic Fas death receptor induction promotes apoptosis resistance in HCC cells

Apoptosis assays were assessed in non-HCC cell (IHH) and HCC cell line (HepG2) as Fas-high-expressing cells. Similar assays were also performed in HCC cells Huh7 and JHH6 as Fas-non/low-expressing cells. Experiments were conducted to observe the effect of Fas exposure in inducing apoptosis after acute (day 1) and chronic stimulation (day 13) as mentioned above.

As shown in Fig. 3.4., for normal IHH cells, at acute treatment on day 1, the percentage of apoptosis, early apoptosis, and late apoptosis were higher in cells treated with anti-Fas (DX2) compared to non-treated cells (CTRL) of Fas exposure using anti-Fas (DX2) in IHH cell. However, the condition was slightly inverted after chronic anti-Fas induction (day 13), as shown in Figure 3.7.

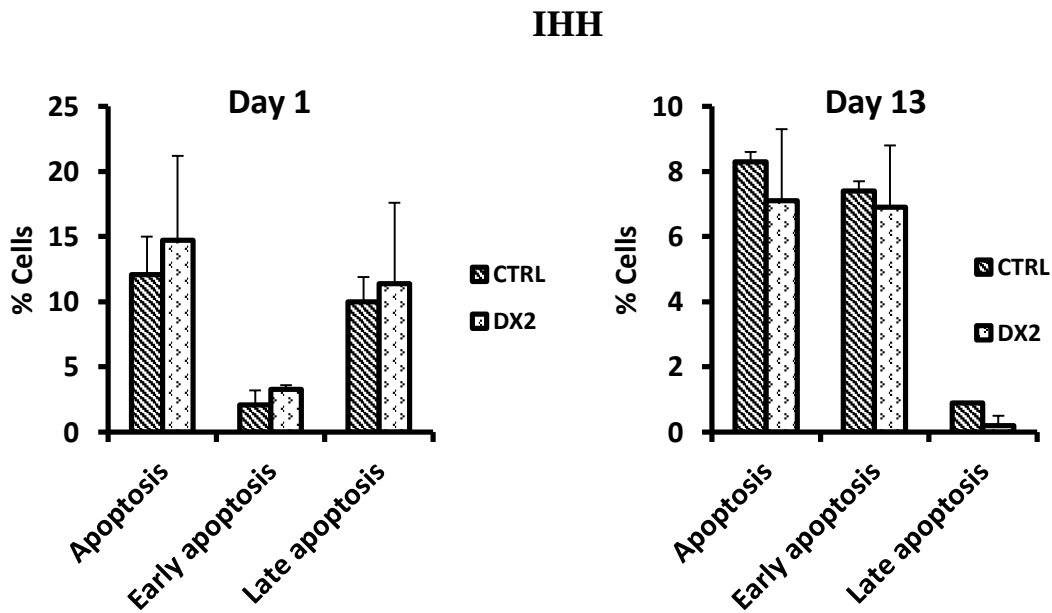


Figure 3.7. Apoptosis signatures after acute and chronic Fas induction in the non-HCC cells IHH.

As shown in Fig. 3.8, for HCC cells HepG2, at acute treatment on day 1, the percentage of late apoptosis was doubled after acute anti-Fas induction (day 1) compared to CTRL. On the contrary, the percentage of apoptosis and early apoptosis were lower after chronic anti-Fas (DX2) induction (day 13) compared to CTRL. Interestingly, the number of late apoptosis was comparable between treatment and CTRL, indicating a possible apoptosis-resistance of these cells in chronic exposure.

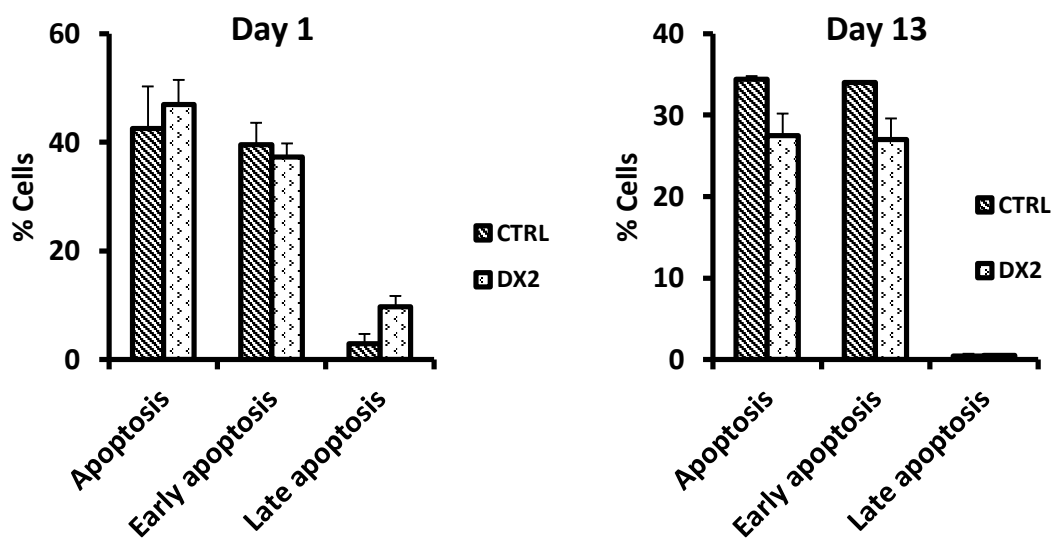


Figure 3.8. Apoptosis signatures after acute and chronic Fas induction in HCC cells HepG2 cell lines.

Surprisingly, we observed also DX2 effect in Fas-low-expressing cell lines. As shown in Fig. 3.9 for HCC cells Huh7, at acute treatment on day 1, the percentage of apoptosis and early apoptosis were higher after acute anti-Fas (DX2) induction (day 1) and chronic Fas induction (day 13) compared to CTRL. As Huh7, in JHH6, the percentage of apoptosis signatures was higher after acute Fas induction (day 1) compared to CTRL (Figure 3.10). After chronic anti-Fas induction (day 13), anyhow, the percentage of late apoptosis was lower compared to CTRL.

From our findings, we could conclude that anti-Fas (DX2) exposure to HCC cells affect cells' resistancy to apoptosis, especially for a longer time exposure.

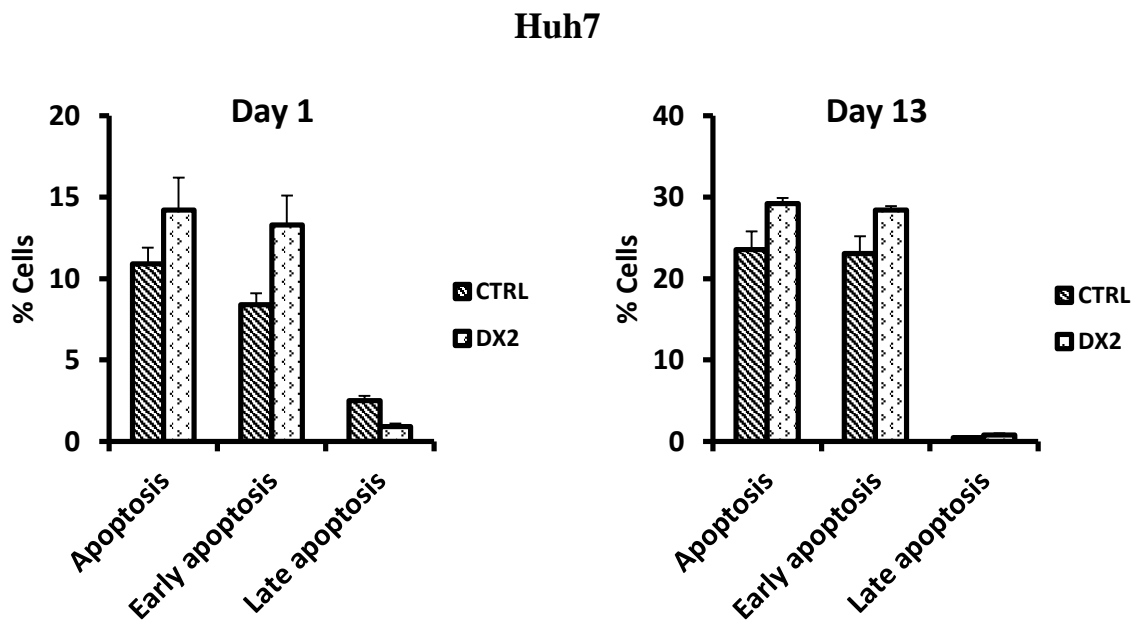


Figure 3.9. Apoptosis signatures after acute and chronic Fas induction in HCC cells Huh7

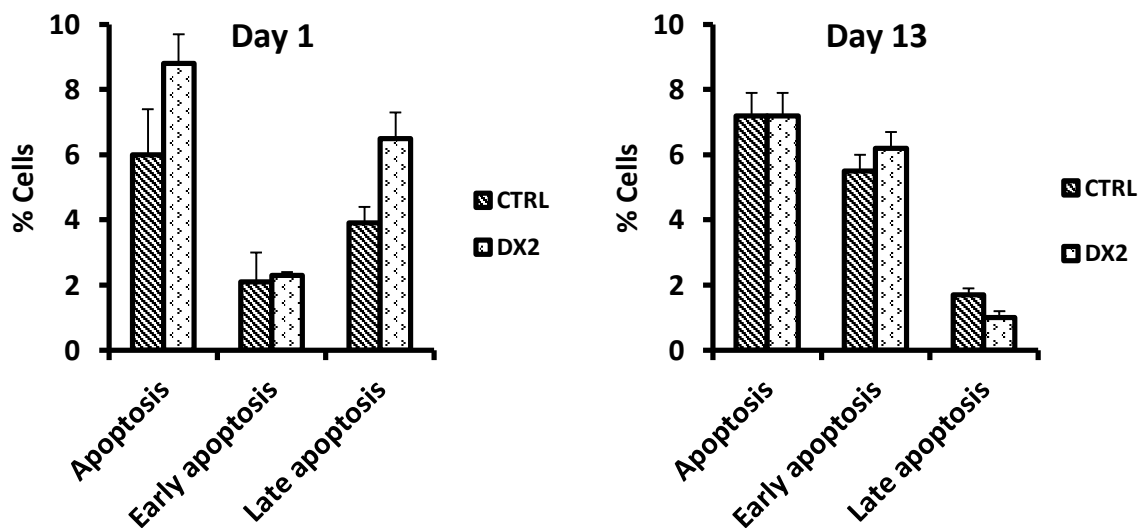


Figure 3.10. Apoptosis signatures after acute and chronic Fas induction in HCC cells JHH6

3.4.4. Deficiency of pro-apoptotic PUMA reduced apoptosis in tumor cell

Our *in vivo* study showed that PUMA mRNA was down-regulated in HCC tissue samples compared to normal tissues. Here, from *in vitro* study, gene expression analysis was performed to determine PUMA expressions anti-Fas (DX2) exposure mentioned above in all cell lines studied.

In acute induction (day 1), we found that the pro-apoptotic PUMA was modulated differently in non-HCC cell lines IHH and HCC cell lines (HepG2, Huh7, and Huh7) after induction by anti-Fas. PUMA mRNA was only up-regulated in normal hepatic cell line IHH, while no changes were noticed in all HCC cell lines, as shown in Figure 3.11.

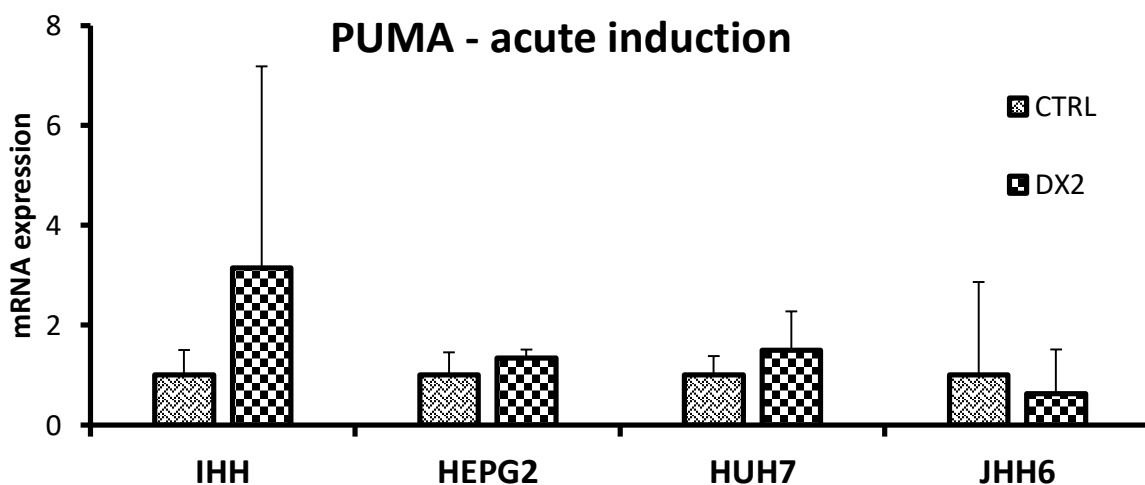


Figure 3.11. The mRNA expressions of PUMA after acute Fas induction in hepatic cell lines.

A more comprehensive analysis was then performed for HepG2. A different situation was observed after chronic Fas induction in this cell. There was a modulation of the PUMA gene expression from day 2 until day 13. After a longer time of Fas induction (day 10), the expression of PUMA mRNA was down-regulated compared to control, as shown in Figure 3.12. The increase of PUMA expression during the culture for the CTRL was due the natural apoptosis process due to the increase of cells number in the plate.

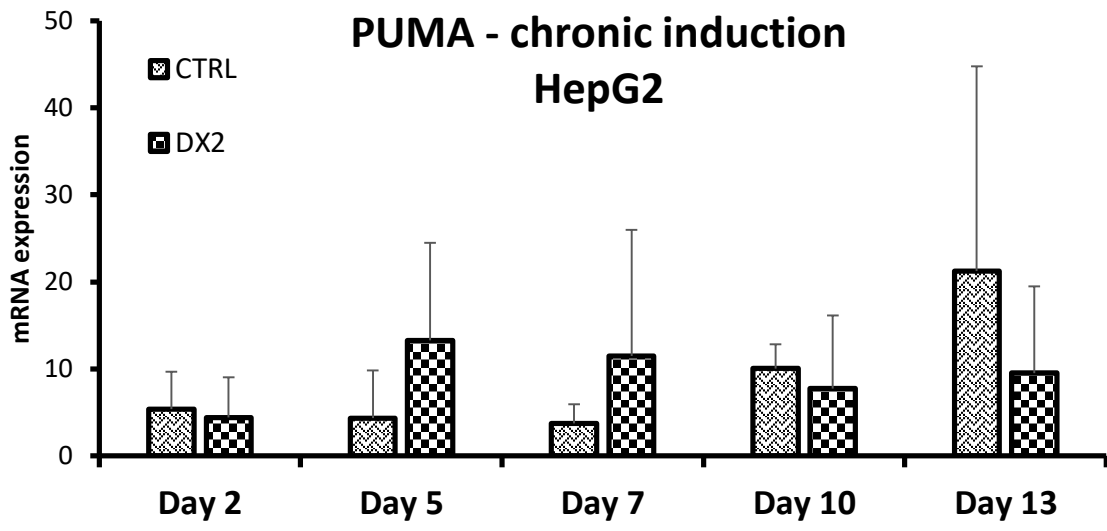
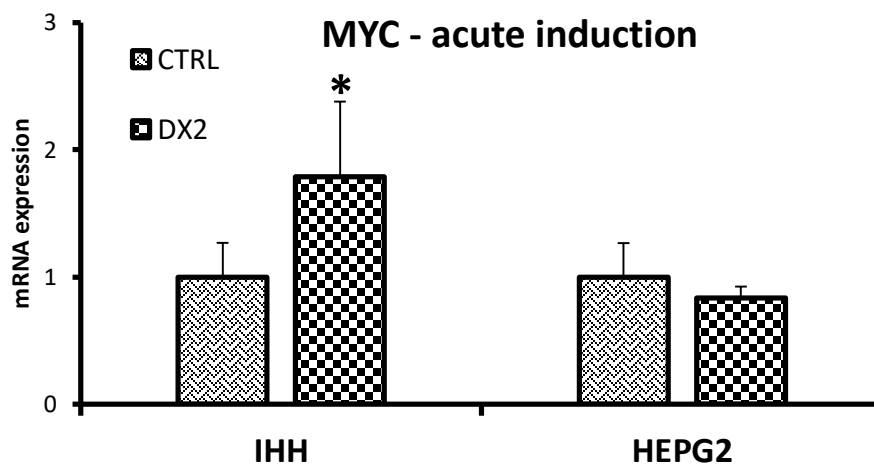


Figure 3.12. The mRNA expressions of PUMA after chronic Fas induction in HepG2.

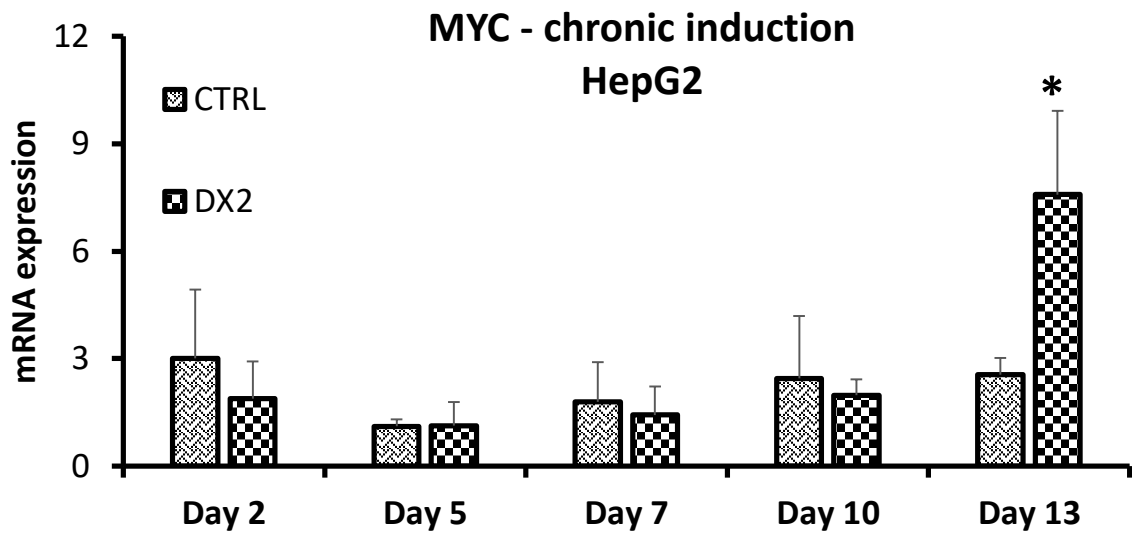
3.4.5. Involvement of mitochondria in Fas-mediated apoptosis

Anti-Fas stimulation activated the intrinsic apoptosis pathway and significantly up-regulated the Myc gene in IHH (Figure 3.13) that was important in mitochondrial apoptosis execution. MYC gene was down-regulated in the HCC cell line probably due to less susceptibility of apoptosis induction by Fas mediated apoptosis. Besides that, other mitochondrial pro-apoptotic protein, PUMA and BAX genes were also modulated in Fas-mediated apoptosis. The results indicated that Fas stimulation also activates the apoptosis-related genes in mitochondria pathway (t-test: * $p < 0.05$ vs CTRL).



*Figure 3.13. The mRNA expression of the MYC gene after acute Fas induction in a non-HCC cells IHH and HCC cells HepG2. (t-test: * $p < 0.05$ vs CTRL)*

Activation of MYC in cancer cells may become a hallmark of cancer maintenance. Since we observed that chronic anti-Fas exposure gave its effect in HepG2, we evaluated MYC expression in chronic induction. In this long anti-Fas induction (day 13), MYC was significantly up-regulated compared with day 2 (Figure 3.14). These results indicated that chronic Fas stimulation activated the MYC gene, which was the hallmark of cancer initiation and preservation.



*Figure 3.14. The mRNA expressions of MYC gene after chronic Fas induction in HepG2 cells. (t-test: *p<0.05 vs day 2)*

3.4.6. PUMA is p53-dependent in Fas-mediated apoptosis

Pro-apoptotic gene PUMA can induce apoptosis either with or without p53 activation. We performed mRNA analysis to observe the cross-link between pro-apoptotic PUMA and transcription factor p53. After acute Fas induction, the mRNA expression of the p53 gene was up-regulated only in the IHH cell line, as shown in Figure 3.15. No modulation of the p53 gene after acute and chronic Fas induction in the HepG2 cell line. These findings indicated that the pro-apoptotic gene PUMA is p53-dependent only in normal cells.

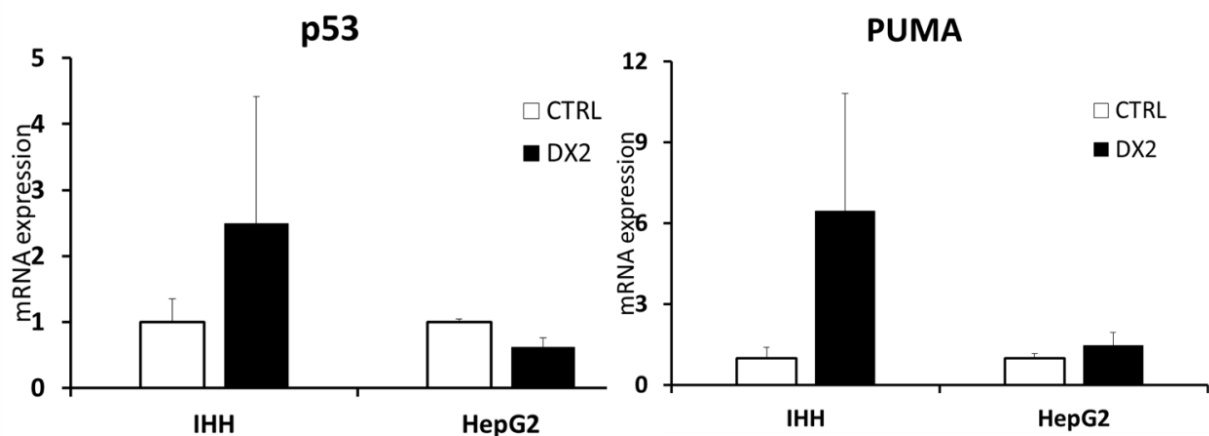


Figure 3.15. The mRNA expressions of p53 and PUMA after Fas induction. The mRNA expressions of PUMA and p53 genes were up-regulated only in normal IHH and not in HCC cells HepG2

3.4.7. Fas increased the cancer stemness after chronic induction

Since recent studies had clearly demonstrated the strong correlation between Fas/FasL and cancer stemness, we checked the expressions of several cancer stem cells (CSC) CD24, CD44, CD133, CD90, and CD13 in the experiments of both acute and chronic anti-Fas (DX2) exposure.

After acute Fas induction, we did not observe any regulations of CSC marker CD24 neither in IHH or HepG2 cells. Even, a slight decrease was noticed in HepG2. Figure 3.16. No modulations were also observed for other CSC marker tested data not shown).

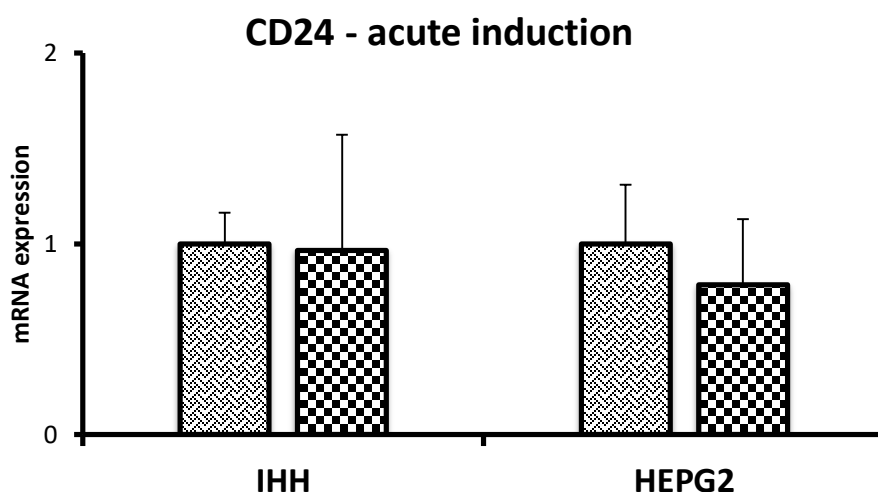


Figure 3.16. mRNA expression of CSC marker CD24 after acute Fas induction in IHH and HepG2

However, we observed that chronic stimulation of the anti-Fas up-regulated the CSC marker CD24 of the HepG2. A significant CD24 mRNA up-regulation was noticed starting from day 10 as seen in Figure 3.17. As expected, the CD24 expression in CTRL was relatively stable.

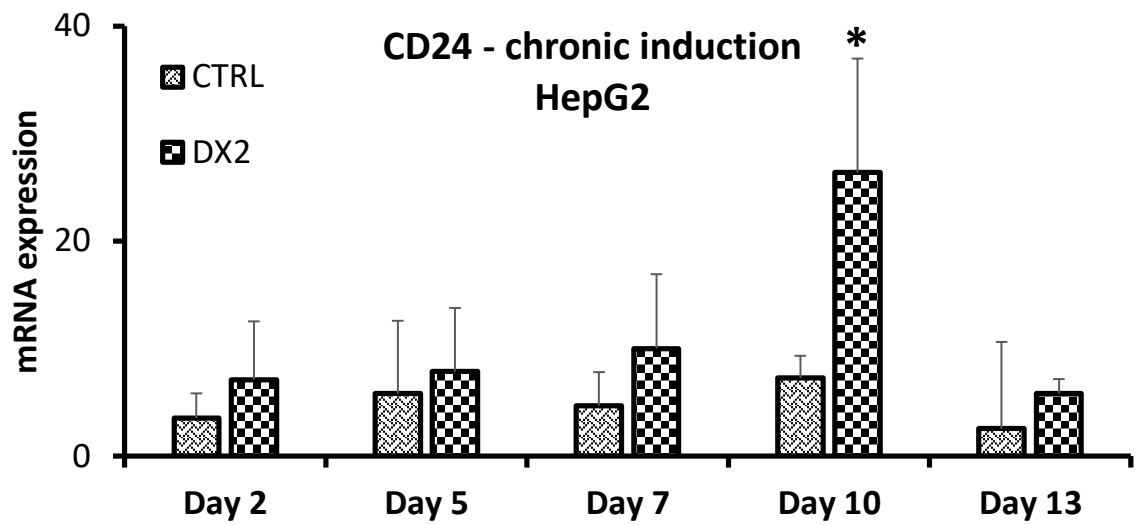


Figure 3.17. mRNA expression of CSC marker CD24 after chronic Fas induction in HepG2. (t-test: * $p < 0.05$ vs Day 2)

3.4.8. TNF- α is important in cancer progression mediated by Fas death receptor

The modulation of TNF- α was noticed in both IHH and HepG2 cells upon acute (day 1) and chronic treatment (day 13). It should be noticed that TNF- α was significantly up-regulated in HepG2 cells after acute Fas induction compared to IHH (Figure 3.18). (t-test: * $p < 0.05$ vs control).

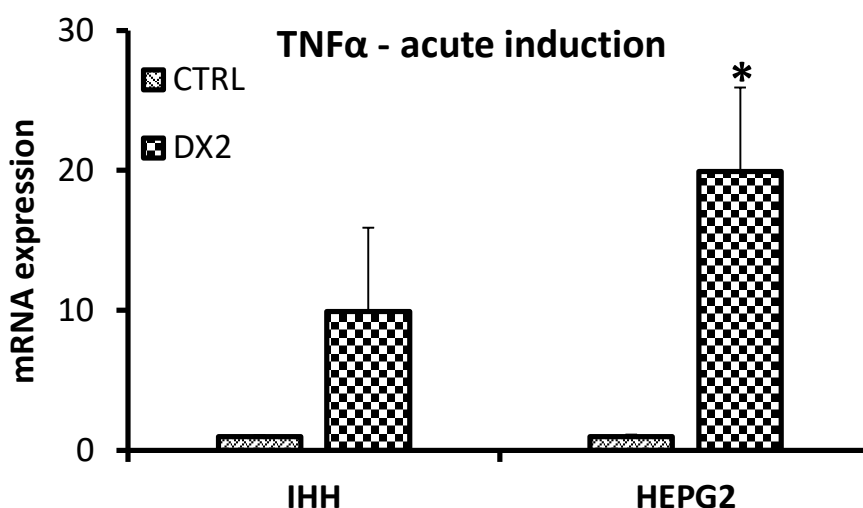
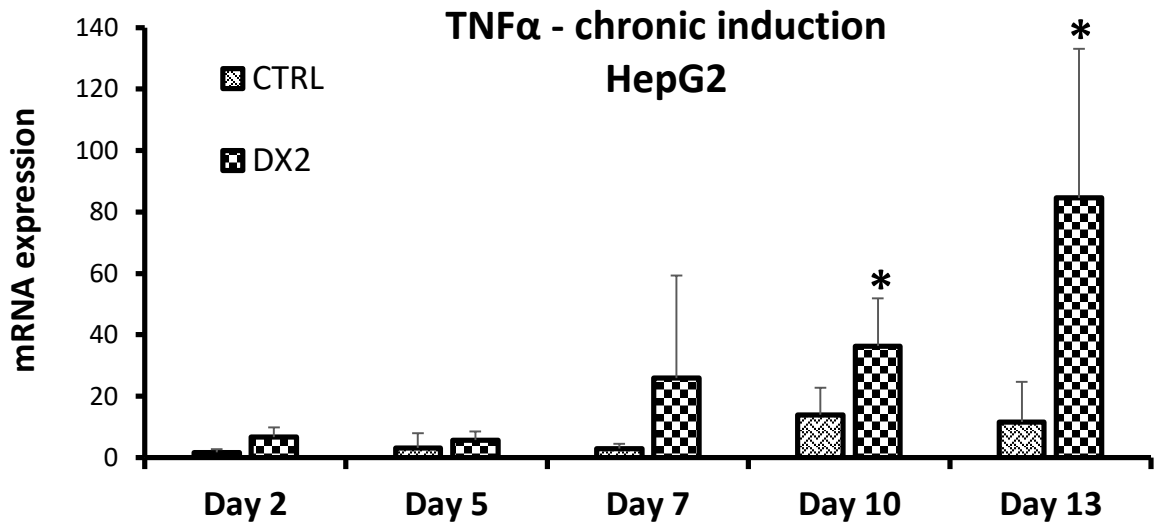


Figure 3.18. mRNA expression of TNF- α after acute Fas induction in the non-HCC cells IHH and HCC cells HepG2. TNF- α was strikingly up-regulated after acute Fas induction in the HepG2 cell compared to control ($p < 0.05$ vs CTRL)*

Further, in HepG2 cells, TNF α was progressively after chronic Fas induction, as shown in Figure 3.19. 6. TNF α was significantly up-regulated on day 10 and day 13 compared to day 2 ($p < 0.05$).



*Figure 3.19. mRNA expression of TNF α after chronic Fas induction in the HepG2 cell line. TNF α was progressively increased in HepG2 cells after chronic Fas induction, to be significant at day 10 compared to day 2 (t-test: * $p < 0.05$ vs day 2).*

3.4.9. NF- κ B promotes cancer development

The transcription factor NF- κ B was variably modulated in HCC and non-HCC cell lines after acute and chronic treatment. Regarding the HepG2, the NF- κ B gene was significantly down-regulated ($p < 0.05$) after acute induction of the anti-Fas compared to CTRL (Figure 3.20).

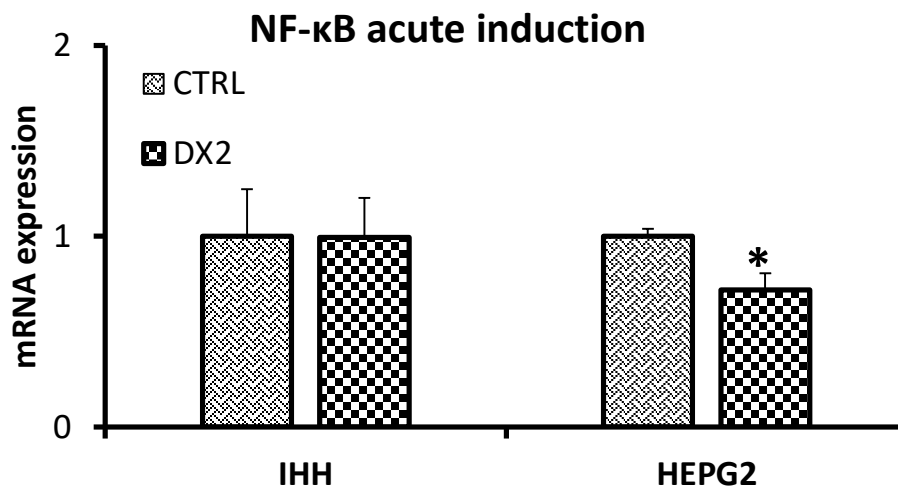


Figure 3.20. mRNA expression of NF- κ B after acute Fas induction in IHH and HepG2. (*t*-test $*p < 0.05$ vs CTRL).

On the contrary, the NF- κ B gene was significantly up-regulated in HepG2 cell after chronic treatment of DX2, again at day 10 and day 13 compared to day 2 as shown in Figure 3.18 (Figure 3.21).

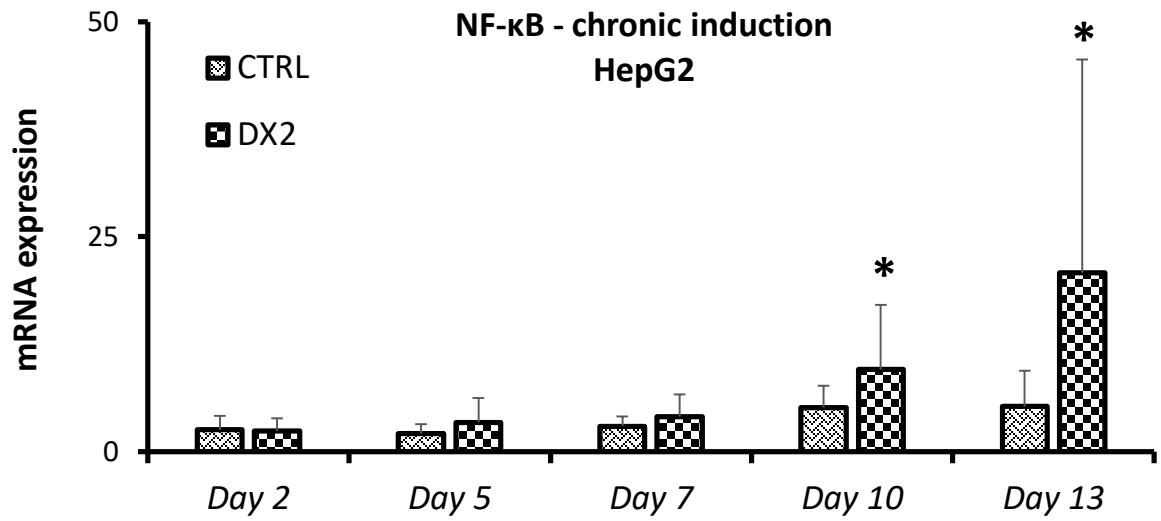


Figure 3.21. mRNA expression of NF-κB after chronic Fas induction in the HepG2 cells. (t-test * $p < 0.05$ vs day 2)

3.5. DISCUSSION AND CONCLUSION

Fas is a cell surface receptor and the key component of extrinsic apoptosis which is important in cellular homeostasis and viral infection. Our study demonstrated that chronic activation by using anti-Fas (DX2) death receptor in HCC cells (HepG2) induced tumor growth. This data supported the evidence on the role the Fas death receptor in cancer resulation and carcinogenesis in in pathological conditions such as the reported breast cancer and glioblastoma studies ^{54,124}. Moreover, we noticed that apoptosis pattern in HepG2 cells to be decreased after chronic stimulation of the anti-Fas. It indicated that the Fas death receptor promoted apoptosis resistance. In clinical cased, this non-apoptotic function might be due to a mutation of the Fas gene. The mutation can eliminate the tumor-suppressive function of Fas and induced the survival pathway¹³².

It is known that the induction of apoptosis by Fas modulates the expression of pro-apoptotic gene PUMA. In our study, after anti-Fas exposure, the up-regulation of PUMA was only noticed in normal hepatocytes (IHH). This indicated that the IHH cell was still susceptible and sensitive with Fas-mediated apoptosis. On the contrary, no significant changes were noticed in HCC cell lines (HepG2, Huh7, and JHH6). This finding indicated that HCC cells were less sensitive or even resistant to Fas-mediated apoptosis. Down-regulation of PUMA will lead to apoptosis deficiency that underlying the increased risk of cancer survival.

Pathological conditions such as cancer are strongly related to apoptosis dysfunction which p53 has a prominent role. Involvement of p53 in apoptosis mechanism could be traced through transcription-dependent and -independent manner ¹³³⁻¹³⁵. In this study, mRNA expression of p53 was down-regulated in HCC cell line concomitant with its target gene PUMA. Loss of p53 can reduce the sensitivity of the cell to Fas-mediated apoptosis ¹²⁶. Activation of p53 induced Puma expression, which can mediate apoptosis either with p53 involvement (p53-dependent) or without p53 involvement (p53-independent) ^{136,137}. Modulation of PUMA indicated that there was an involvement of mitochondria and p53 in Fas-mediated apoptosis, suggesting PUMA is p53-dependent.

In this study, we observed that HCC might have resistant mechanisms against Fas-mediated apoptosis. This capability might correlate with other signatures that were also activated

after the Fas induction. We discovered that MYC was significantly up-regulated after chronic Fas induction. MYC is a powerful apoptosis inducer via a mitochondrial pathway through cytochrome *c* release with the intervention of p53 and Fas/FasL signaling¹³⁸. Regarding our findings related to the chronic Fas induction as a tumor growth inducer and apoptosis-resistant promoter, MYC was mostly contributed as an oncogene that is essential in the pathogenesis of human cancers. Activation of MYC after chronic Fas induction might become a hallmark of HCC preservation. MYC dictates tumor cell fate, by blocking the cellular senescence, orchestrating changes in the tumor microenvironment, and inducing stemness¹³⁹.

Fas play a pivotal role in cancer defense and recurrences by counterattack the immune system and increase the stemness of the tumor cells in several cancer^{50,124}. In our study, chronic Fas induction significantly up-regulated the cancer stem cell marker CD24. In several cancers, such as breast cancer and glioblastoma, Fas was also responsible for inducing cancer stemness⁵¹. The collected data clearly suggested that chronic stimulation of the Fas death receptor increased the cancer stemness of HCC.

Another interesting finding from our study was the activation of TNF α and NF- κ B transcription factors after chronic Fas induction. Although primarily TNF α thought to give effect as an anti-tumor signature, in recent years, it has been discovered that TNF α was also responsible for promoting tumor survival. It has diverse roles in cancer metastasis that is crucial for the tumor progression^{63,64}. In this study, we observed a significant up-regulation of intracellular TNF α after both acute and chronic Fas induction in the HCC cells. This modulation is probably related to the non-apoptotic function of Fas that activated the TNF α , which is known as a key factor of tumor survival that can enhance tumor growth and metastasis¹⁴⁰. The result was supported by the result of late apoptosis and the activation of the NF- κ B transcription factor. TNF α increases the capability of invasion and metastasis through the NF- κ B signaling pathway¹⁴¹.

NF- κ B transcription factor not only has an essential role in regulating gene for cell survival but also play a fundamental role in tumorigenesis of many human cancer¹⁴². Since the role of the NF- κ B transcription factor in HCC is not well studied, this finding may represent a new pathway or mechanism in tumorigenesis of HCC. NF- κ B was modulated early after Fas stimulation and the signature change upon long-term stimulation⁵⁰. We demonstrated that the NF-

κ B signature was significantly increased after chronic Fas death receptor induction. The up-regulation of NF- κ B may suppress the apoptotic response and promoted tumor survival.

NF- κ B established as a crucial contributor in metastasis, cell proliferation, anti-apoptosis, and angiogenesis of the malignant tumors^{143,144}. We finally discovered that Fas promote cancer survival and tumorigenesis through the activation of the TNF- α /NF- κ B pathway after chronic stimulation.

Overall, our study strongly suggests the involvement of mitochondria and p53 in Fas-mediated apoptosis of HCC. These findings revealed a complex cross-talk between extrinsic apoptosis pathway and mitochondrial pathway. Utilizing *in vitro* model, we identified that Fas death receptor are responsible in tumor growth, apoptotic resistance, cancer survival and cancer stemness in HCC.

In conclusion, our findings represent a mechanism that underlying the apoptosis resistance in HCC cells. This study revealed that there was a link between the Fas death receptor and MYC/ TNF- α / NF- κ B pathway. Molecular signatures involved in the interplay between these pathways and difference behaviors between non-HCC and HCC cells after Fas induction identified promising new targets for therapeutic intervention. The essentials of chronic Fas death receptor induction in inducing the tumor growth, supporting cancer preservation, and promoting the cancer survival and tumorigenesis is the main reason to develop an oligonucleotide nanomedicine against Fas death receptor.

Chapter 4

**DEVELOPMENT OF APTAMER:
OLIGONUCLEOTIDE NANOMEDICINE
AGAINST FAS DEATH RECEPTOR IN
HEPATOCELLULAR CARCINOMA**

4. DEVELOPMENT OF APTAMER: OLIGONUCLEOTIDE NANOMEDICINE AGAINST FAS FOR HCC

4.1. BACKGROUND

In HCC, oligonucleotide nanomedicine therapy was predicted to achieve a better result than antibody-based therapy due to the non-effective treatment of the tested drug, codrituzumab (antibody-based therapy) against HCC^{145,146}. As mentioned above, the aptamer is a potent tool in basic and clinical biomedicine. Until now, numerous aptamers for different biomedical applications as biosensor and imaging nanoparticle for diagnostic¹⁴⁷⁻¹⁴⁹, drug delivery agent¹⁵⁰⁻¹⁵², and theranostic (therapy and diagnostic)¹⁵³⁻¹⁵⁵ had been discovered. Since Pegaptanib, an aptamer targeting vascular endothelial growth factor (VEGF), had been approved for age-related macular degeneration treatment¹⁵⁶, several aptamers had been shown to be a promising tool in clinical applications. Aptamer AS1411 (for acute myeloid leukemia and renal cell carcinoma) and NOX-A12 (for chronic lymphocytic leukemia and refractory multiple myeloma) are currently used in clinical trials^{157,158}. AS1411 was shown to selectively recognize cancer cells *in vivo* without any major side effects and toxicity¹⁵⁹. Meanwhile, several aptamers are still under preclinical trials.

The increasing number of cases and poor prognosis of HCC highlight the need for a significant, appropriate, and efficient management of the disease. The screening and verification of potential aptamers as molecular probes against HCC will be needed to discover novel biomarkers in diagnostic and therapeutic implications¹⁶⁰.

Oligonucleotide aptamer is an emerging and promising nanomedicine for HCC diagnosis and therapy in the future. Aptamer can be a powerful tool with unique and distinctive characteristics that will give positive impacts, both in basic research and clinical application of HCC. We predict that in a near future aptamer technology will continue to exponentially grow and to be increasingly used in the development of new efficacious aptamer-based tools for diagnosis, nano-delivery, biosensor and therapeutic agents towards cancers, including HCC.

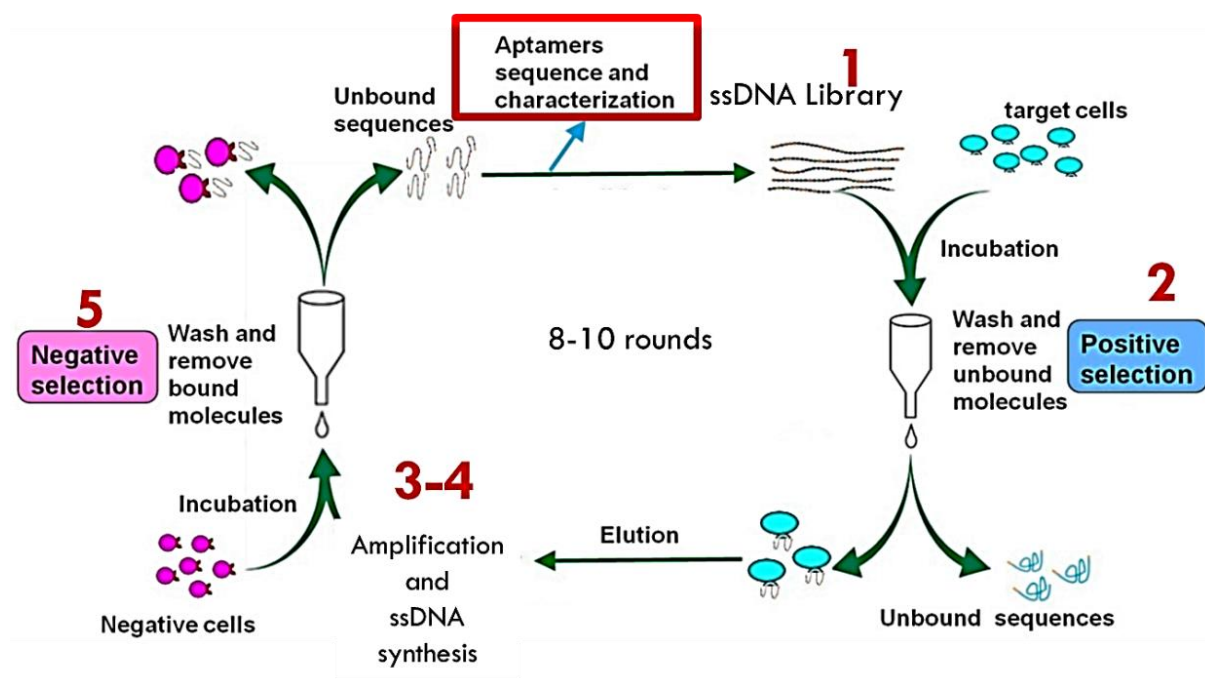
4.2. AIM

Fas death receptor is the key point of the extrinsic apoptosis pathway that play a pivotal role in tumor growth and cancer stemness that lead to the therapeutic resistance and recurrence. Fas is a promising target that is important in developing novel theranostic (therapy and diagnostic) against many types of cancer, especially HCC. The principal aim of this study is to discover an oligonucleotide aptamer against Fas death receptor for HCC.

4.3. MATERIAL AND METHODS

4.3.1. Cell-Systematic evolution of ligands by exponential enrichment (Cell-SELEX)

The aptamer can be generated by using Systematic Evolution of Ligands by Exponential Enrichment (SELEX). In this study, Cell-SELEX was performed until 10 cycles with several modifications and adjustment. Number of cells, aptamer ratio, length and condition of incubation and PCR amplification optimization were optimized in each cycles of selection.



Systematic Evolution of Ligands by Exponential Enrichment (SELEX)

DNA library

Oligonucleotide DNA aptamer was developed using a random DNA library. The 40 nt DNA library was purchased from a commercial manufacturer (TriLink BioTechnologies, Sanm Diego, CA, USA). In brief, the DNA library was suspended in 100 ul nuclease-free water to reach 100uM concentration. The working concentrion used for the DNA library is 100 nM for PCR of the SELEX protocol.

The PCR optimization was conducted by using two sets of primers (TriLink - blank P0-F and P0-R, and Metabion – with fluorescein and biotin P0-DNA-F FITC and P0-DNA-R- Biotin). PCR reaction was conducted based on TriLink protocol with denaturation: 95°C for 5 min; annealing: 95°C for 30 sec, 50°C for 30 sec, 72°C for 30 sec for 25 cycles; extension: 72°C for 5 min; Hold at 4°C. 4% agarose gel was used to run 10 ul PCR product to check the DNA band.

Table 4.1. The PCR reaction mix

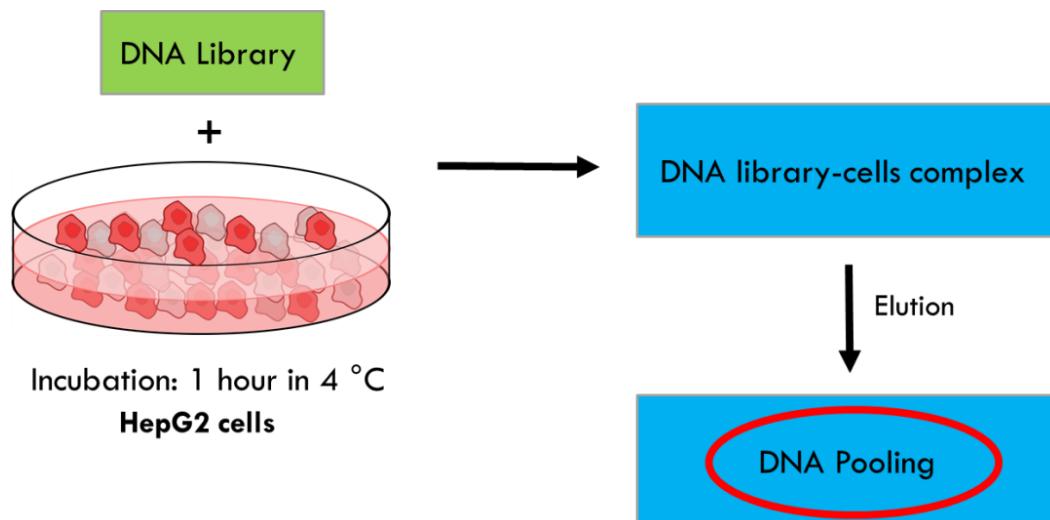
Reagents	Stock conc.	Rx conc.	ul/ Rx	Rx mixture x3
10x PCR buffer	10x	1x	2.5	7.5
dNTP	25 mM	0.2 mM	0.2	0.6
MgCl ₂	50 mM	1 mM	0.5	1.5
Primer F	100 uM	2 uM	0.5	1.5
Primer R	100 uM	2 uM	0.5	1.5
DNAase-free water			19.55	58.65
Taq Polymerase	5 U/ul	0.05 U/ul	0.25	0.75
DNA library 100 nM	100 nM	4 nM	1	

Cell preparation

HCC cell lines HepG2 (Fas-expressing cells; Positive selection) and HUH7 (Fas-non-expressing cells; Negative selection) were used in the Cell-SELEX method. Both of cell lines were cultured on a 100-mm sterile dish until reaching 90% confluence. The cells were washed twice using washing buffer before proceeding the selection. In the first round of Cell-SELEX, the HepG2 cells were incubated with the random DNA library (positive selection).

Positive selection

Positive selection was performed using HepG2 cells that have more than 50% of Fas receptor. The cells were cultured in 100-mm culture dish until 90-100% confluence. 60 μ l of 0.5 mM (10 nmol) DNA library was added to 1050 μ l of binding buffer. The mixture was mixed and denaturalized by heating at 95 $^{\circ}$ C for 5 minutes. An immediate cooling was then performed on ice for 10 minutes to reduce intermolecular hybridization. Cells were then incubated with the DNA library with total volume of 1110 μ l (DNA library + binding buffer) for a 100-mm dish. Continuous incubation was done on an orbital shaker (50 rpm) or on slow rocker for 1 h in cold room. After the incubation, the dish was washed and treated with gradual concentration of DNase (50U, 100U and 150U) to remove unbound sequence

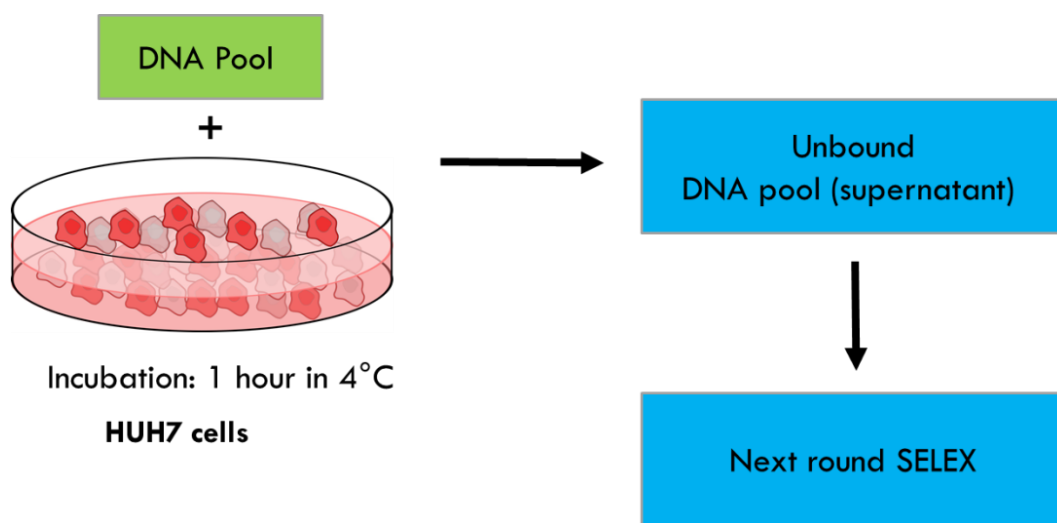


The cell-DNA complex was then re-suspended with 500 μ l of DNase-free water and detached using cell scraper and transferred into a 1.5-ml tube. The cell-bound ssDNA was eluted by heating at 95 $^{\circ}$ C for 10 min to disrupt the interaction between the DNA and the cell surface receptors. The cells-DNA complexes were centrifuged at 13,100g rpm for 5 min in room temperature to precipitate the cell debris. The supernatant that containing eluted DNA was

collected and transferred into a new tube and labeled as Pool 1. The eluted DNA was stored at – 20 °C to be used for negative selection.

Negative selection

Negative selection was performed in every cycle of Cell-SELEX. Huh7 cells (Fas negative cells) were used in negative selection. The cells were cultured in 100-mm culture dish until 90-100% confluence. The growth medium was removed and cells washed with washing buffer. The cells were then incubated with the ssDNA from the positive selection above in cold temperature with continuous shaking for 1 hour. After the incubation, the buffer containing unbound sequences to the control cells was recovered for the next cycle of selection. The selected DNA pool was labeled according to the Cell-SELEX cycle.



PCR amplification

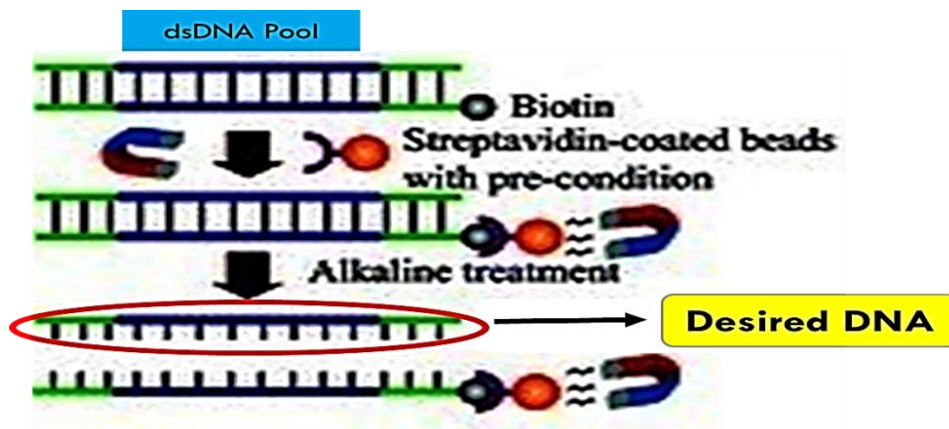
PCR amplification was performed in every selection cycle to find the optimal number of cycle to amplify the DNA. The PCR reaction were using 5x Phusion HF buffer (Thermo Fisher), 25 mM dNTP mixture, 10 uM of forward and reverse-Biotin primers (Metabion), 2 U/ul Phusion HS II Polymerase (Thermofisher), DNase-free water and DNA pool 1-10. The PCR condition

were 98°C for 10 s, 57°C for 30 s and 72°C for 30 s for 10-30 cycles. The concentration of the PCR product (dsDNA) was measured by calculating the absorbance at 260 nm in a DU730 spectrophotometer (Beckman Coulter, Fullertone, CA, USA).

ssDNA synthesis

The amplified DNA was synthesized into ssDNA for the next cycle of selection taking advantages of biotin-conjugated of the primer. We used the he Dynabeads® MyOne Streptavidin C1 (Invitrogen) system on magnetic particle concentrator-Dynal MPC-S (DynaL Biotech).

The biotin-labeled double-stranded (ds) PCR product was incubated with Dynabeads for 2 h at room temperature. The biotinylated DNA coated beads were then separated with the magnet by incubating the complex for 3 minutes in room temperature. The non-biotinylated ssDNA were transfer to a new tube and the concentration of the ssDNA was measured using Spectrophotometry.



4.3.2. ssDNA and Fas-His Tag protein precipitation

As control, we used Fas human recombinant protein (Sino Biological Inc., Beijing, China) to discover the sequence of the aptamer that perfectly binds to Fas protein. After 10 cycles of Cell-SELEX, we used the ssDNA of Pool 10 was precipitated with Fas human recombinant.

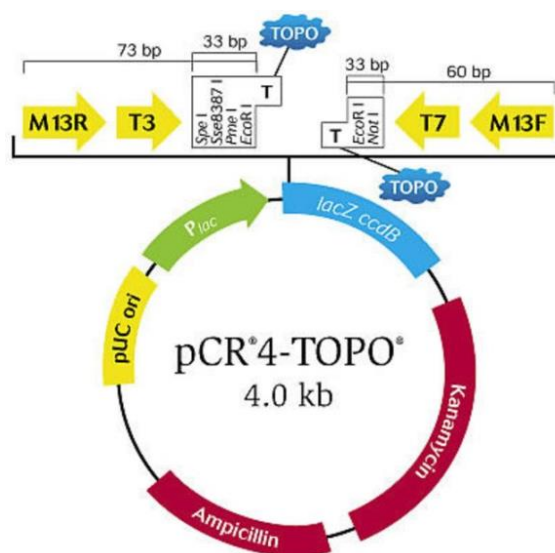
300 pmol of pool 10 ssDNA aptamer was incubated with 2.5 μg of human Fas recombinant protein in binding buffer. The incubation was performed at 4°C with slow rotate for two periods: 2 hours and overnight. After the incubation, the tube was placed on the magnet for 30 minutes, following by supernatant discard. The beads were then washed 4 times with 1X binding buffer by placing the tube on a magnet for 2 min. 250 μl of His-Elution Buffer was added before the suspension was incubated on a shaker for 30 min at 4°C. Finally, the tube was applied on the magnet for 30 minutes and the supernatant containing the eluted histidine-tagged protein was transferred into a clean tube for cloning and sequencing.

4.3.3. Cloning

The cloning experiments were performed using TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA, USA). Fresh PCR products from pool 10 of Cell-SELEX were used for cloning. Before cloning, PCR products were incubated with 0.7-1 Unit of *Taq polymerase* for 8-10 minutes at 72°C for adding the poly- 3' A.

The cloning reaction was performed using 0.5-4 μl of fresh PCR product, 1 μl of salt solution, 1 μl of TOPO vector and water. The cloning reaction were mixed and incubated 5-30 minutes at room temperature. After the incubation, 2-4 μl of the cloning reaction was transformed into the One Shot Competent cells and incubated on ice for 5-30 minutes. The cells were heat-shock for 30 seconds at 42°C and they were immediately transferred the tubes on ice. 250 μl of room temperature S.O.C. medium were added into the cells before shake horizontally at 37°C for 1 hour. 10-50 μl of transformation mixture were spread on a pre-warmed selective plate. The plates were incubated at 37°C overnight.

The growth colonies were picked and re-cultured in LB medium for 24 hours. The re-cultured colonies then purified by using mini prep plasmid purification kit (Euro Clone) following the manufacture's protocol. The DNA plasmids were amplified using the primers provided in the kit (M13R, M13F, T7 and T3). Purified plasmids were sent for sequencing.



4.3.4. Sequencing analysis

For the first analysis, DNA sequencing was performed for representative clones by using T7 primer of the TOPO plasmid. The DNA concentration of the clones obtained after minipreparation was measured by spectrophotometer. The ratio A260/A280 was considered for the good quality of the samples. For the sequencing, DNA amount of 250 – 600 ng was subjected for the sequencing.

The sequencing data was analyzed by using NCBI's BLAST and Bioedit program (Copyright Tom Hall, Ibis Theurapeutics, Carlsbard, CA, USA) to select the candidate of aptamer. The mfold program was then used for further analysis.

4.4. RESULTS

4.4.1. DNA library optimization

The random DNA library was optimized according to the manufacturer protocol (TriLink) to obtain a single band before starting the first round of the cell-SELEX (Figure 4.1). In this optimization, a 25-cycles PCR was chosen.

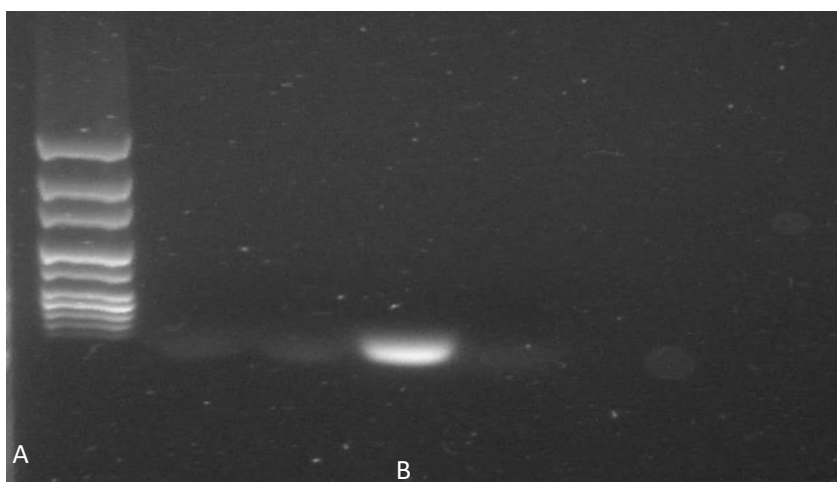


Figure 4.1. Agarose gel electrophoresis image showing the random DNA library optimization. A= 100-bp ladder; B= DNA library with 25 cycles of PCR

4.4.2. Cell-SELEX

Ten continuous rounds of positive and negative selections was performed for Cell-SELEX with modifications. The number of PCR cycles performed was started at 10-cycle for the first SELEX round, and it was then optimized for 10-30 cycles for the sequent rounds. It was done to obtain the optimal cycles with a single and good DNA band as (Table 4.1. and Figure 4.2). The dsDNA were synthesised to ssDNA for the next round of selection (Table 4.2.).

Table 4.1. Number of optimal cycles of PCR and dsDNA pool concentration

Cell-SELEX (round)	Optimal number of amplification cycles	dsDNA concentration
Round 1	10 cycles	0.758 $\mu\text{g}/\mu\text{L}$
Round 2	14 cycles	0.495 $\mu\text{g}/\mu\text{L}$
Round 3	16 cycles	0.510 $\mu\text{g}/\mu\text{L}$
Round 4	20 cycles	0.415 $\mu\text{g}/\mu\text{L}$
Round 5	20 cycles	0.633 $\mu\text{g}/\mu\text{L}$
Round 6	22 cycles	0.508 $\mu\text{g}/\mu\text{L}$
Round 7	22 cycles	0.528 $\mu\text{g}/\mu\text{L}$
Round 8	22 cycles	0.675 $\mu\text{g}/\mu\text{L}$
Round 9	24 cycles	0.893 $\mu\text{g}/\mu\text{L}$
Round 10	30 cycles	0.728 $\mu\text{g}/\mu\text{L}$

Table 4.2. ssDNA concentration after dsDNA synthesis

Cell-SELEX	dsDNA concentration	ssDNA concentration
Pool 1	0.758 $\mu\text{g}/\mu\text{L}$	0.056 $\mu\text{g}/\mu\text{L}$
Pool 2	0.495 $\mu\text{g}/\mu\text{L}$	0.066 $\mu\text{g}/\mu\text{L}$
Pool 3	0.510 $\mu\text{g}/\mu\text{L}$	0.063 $\mu\text{g}/\mu\text{L}$
Pool 4	0.415 $\mu\text{g}/\mu\text{L}$	0.071 $\mu\text{g}/\mu\text{L}$
Pool 5	0.633 $\mu\text{g}/\mu\text{L}$	0.155 $\mu\text{g}/\mu\text{L}$
Pool 6	0.508 $\mu\text{g}/\mu\text{L}$	0.084 $\mu\text{g}/\mu\text{L}$
Pool 7	0.528 $\mu\text{g}/\mu\text{L}$	0.018 $\mu\text{g}/\mu\text{L}$
Pool 10	0.728 $\mu\text{g}/\mu\text{L}$	0.144 $\mu\text{g}/\mu\text{L}$

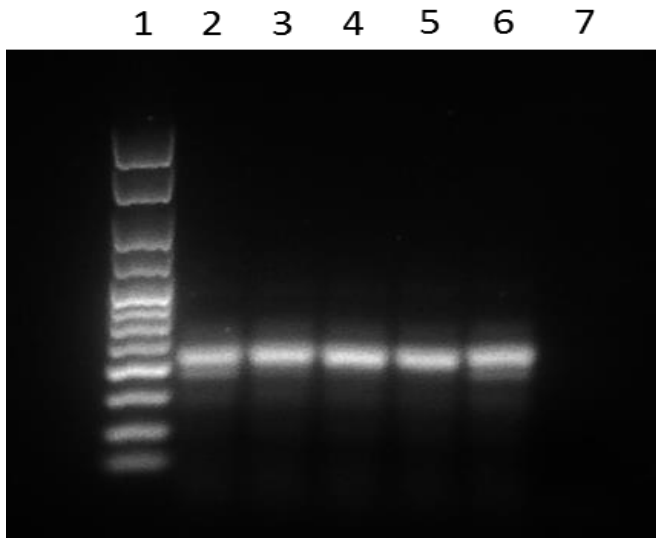


Figure 4.2. Agarose gel image of the products of the various cycles of selected DNA Pool. Lane 1 = 25-bp ladder; lane 2 = 18 cycles; lane 3 = 20 cycles; lane 4 = 22 cycles; lane 5 = 24 cycles; lane 6 = positive control; and lane 7 = negative control

4.4.3. Oligonucleotide screening by cloning and sequencing

Cloning experiments were performed in a TOPO-TA™ cloning system to amplify and to screen the pool DNA aptamer target. Positive control of the kit was tested due to test the efficiency of the vector, reagents and the competent cells. We obtained a total of 72 colonies achieved from the positive control (Figure 4.3). Cloning optimization was done to find the equal ratio of the vector, the insert, and the TOPO reaction. For the aptamer pool, we obtained a total of 68 colonies achieved fresh DNA insert (from cell-SELEX HepG2/Huh7 and Fas recombinant protein) using several competent cells (Figure 4.4).

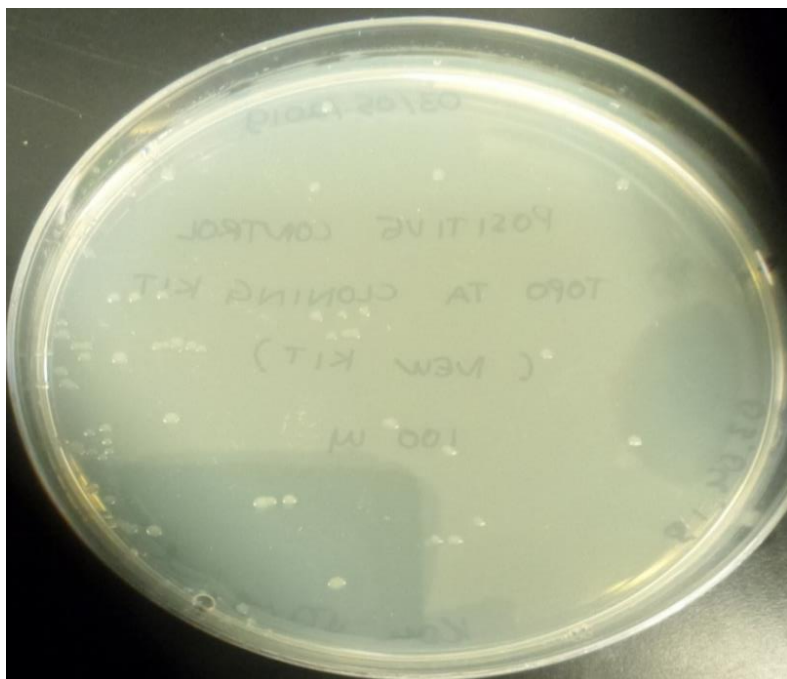


Figure 4.3. Colonies of positive control in LB agar with 100µg/ml of ampicillin

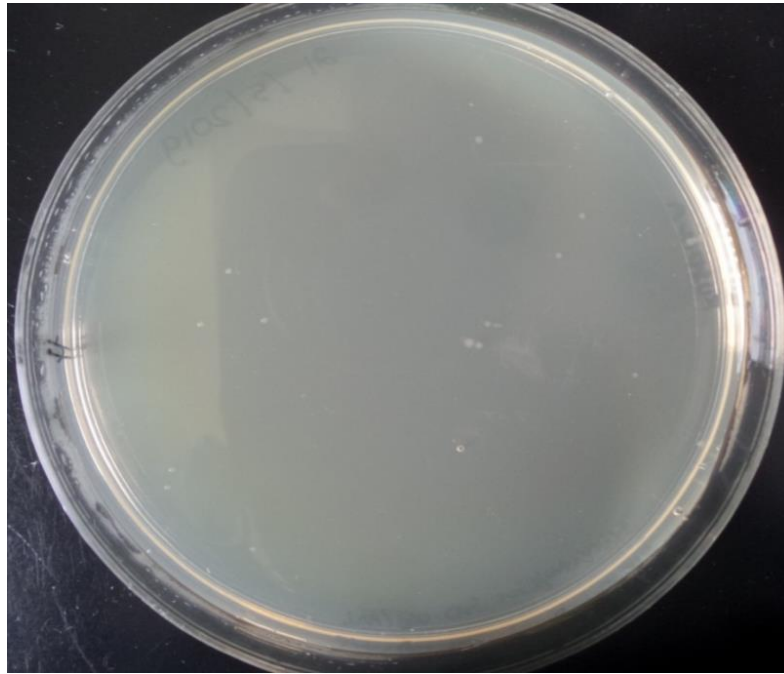
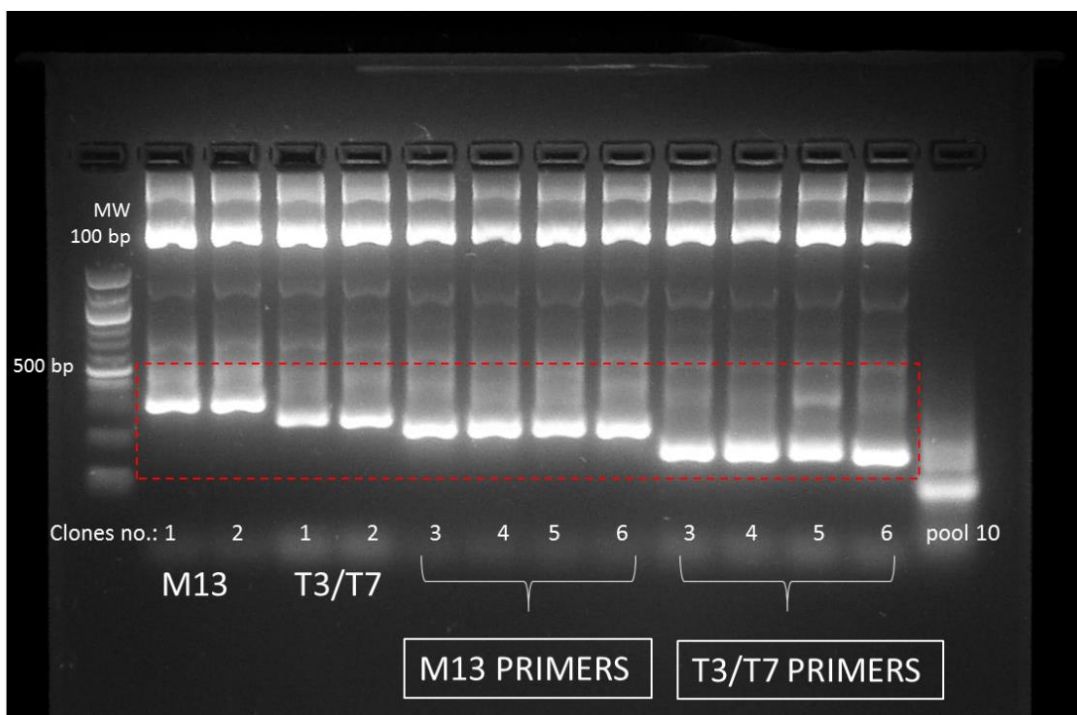
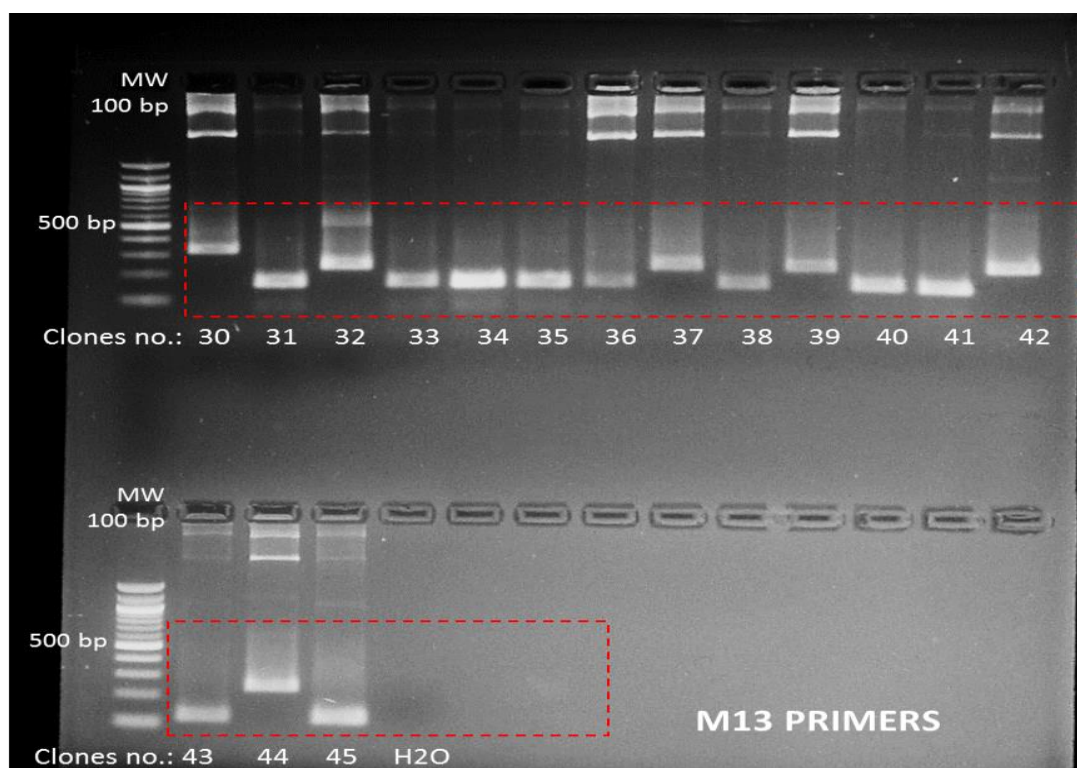


Figure 4.4. Colonies of Cell-SELEX Pool 10 in LB agar with 50µg/ml of kanamycin

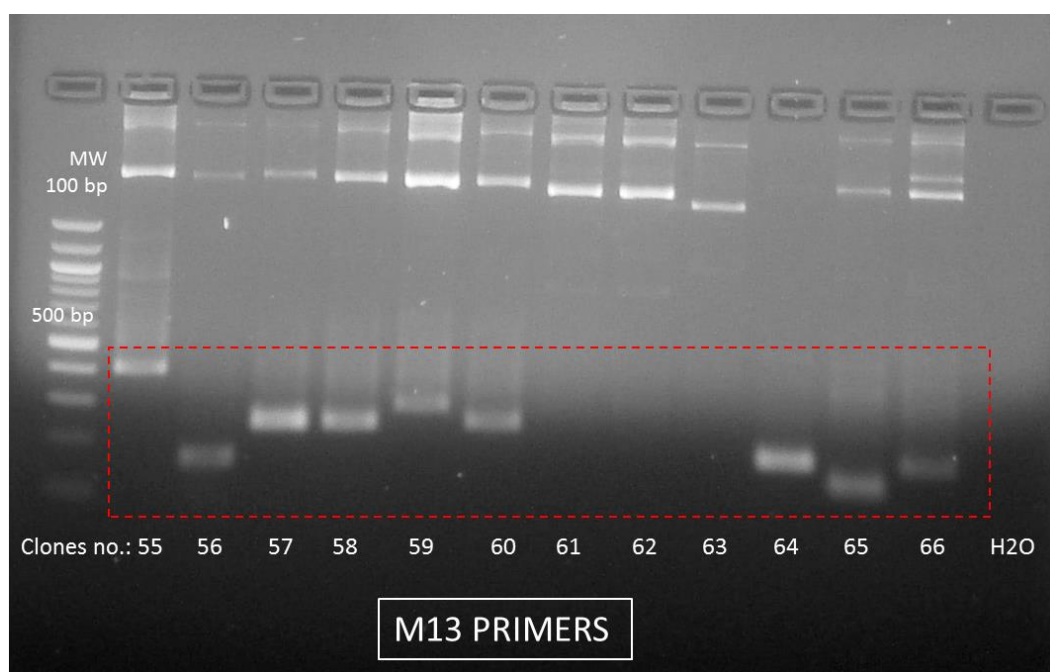
The colonies were purified by DNA plasmid purification kit based on the manufacturer protocol (EuroClone) and amplified using standard primers M13, T3 and T7 primers before sent for sequencing (Figure 4.5).



A



B



C

Figure 4.5. DNA plasmid detection for sequencing analysis. DNA plasmids were amplified with primer M13-forward and reverse, T3 and T7 before sent for sequencing to detect the quality of DNA. Only DNA plasmids with good quality were sent for sequencing. (A) Clones 1-6 were tested using M13, T3 and T7 primers. (B) Clones 30-45 were tested using M13 primers. (C) Clones 55-66 were tested using M13 primers. Red squares: candidates for aptamer.

4.4.4. Sequencing analysis

The sequencing data was analyzed by using NCBI's BLAST and Bioedit program to get the candidate of aptamer. For the first analysis, we performed DNA sequencing of 40 plasmid from 40 bacterial clones. In this preliminary analysis, DNA sequences that frequently appeared with high percentages (around 90%) was selected as the aptamer candidate. The putative candidates for aptamer was labeled as RBL1. Figure 4.6 showed the predicted structure of RBL1 aptamer using *mfold* program.

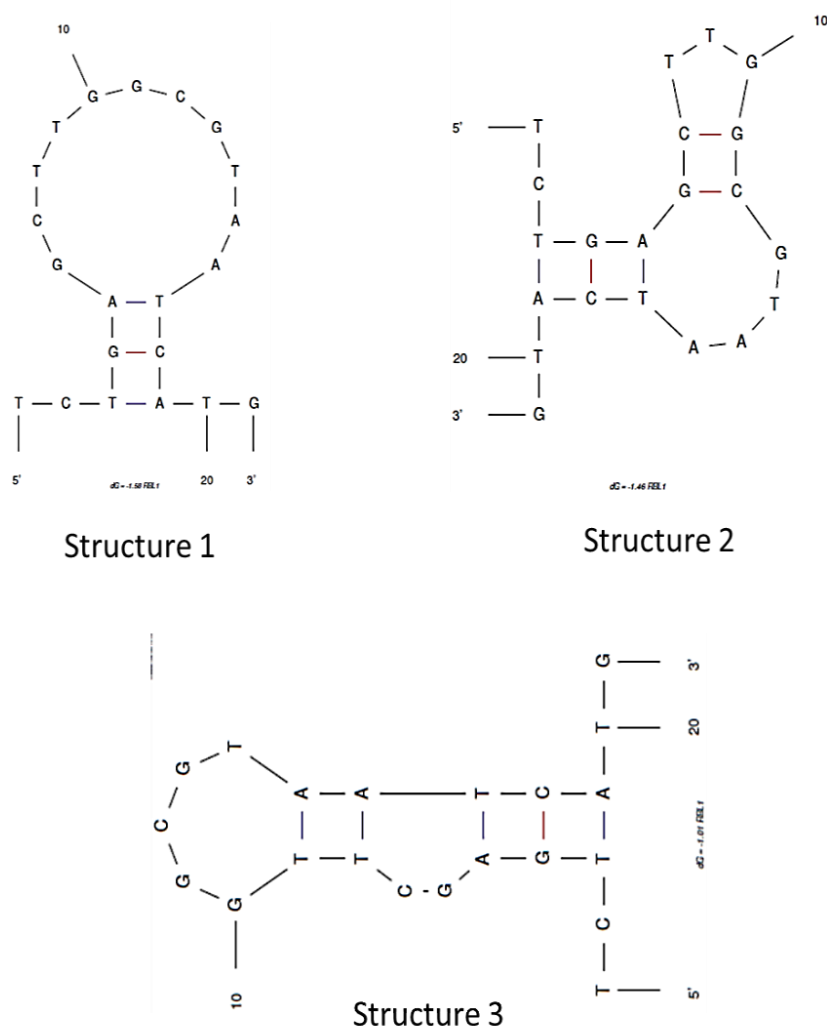


Figure 4.6. Predicted structures of the putative aptamer candidates. The selected DNA was folded to predict the structure of the aptamer. They has 3-folded structures that was predicted by using *mfold* program.

4.5. DISCUSSION AND CONCLUSION

Hepatocellular carcinoma (HCC) is one of the most common cancers with a high mortality rate. Late diagnosis and poor prognosis are still a major drawback since curative therapies such as liver resection and liver transplantation are effective only for an early stage of disease. HCC treatment options are still hampered by many obstacles. Development of oligonucleotide nanomedicine as an early diagnostic tools and novel molecular targeting therapies will be crucial to improving survival rate and life quality of the patient.

Aptamer is an oligonucleotide nanomedicine that has high capability to bind the target molecules in a way similar to antibodies. Aptamer has more advantages over antibody in terms of low/no immunogenicity, longer shelf life, more stable and has low batch to batch variation¹⁶¹. The process of aptamer selection is lengthy and the validation will be needed in different sets of samples and models. Therefore, a target molecule must be carefully selected for a specific and effective approach.

The identification of aptamer against whole cells is called Cell-Systematic Evolution of Ligands by Exponential Enrichment (SELEX). The process consisting of several phase of selections that including of binding phase, partitioning phase, elution phase, amplification, and screening phase. By using Cell-SELEX technique, identification of aptamer for targeted receptor was performed in its natural environment where the target in its native conformation and in the same environment. In that way, the aptamer development is one step closer to the clinical translation.

According to our previous study, we concluded that Fas death receptor is one the key components of HCC pathogenesis and development. In this chapter, we did the establishment and the of an oligonucleotide nanomedicine against the Fas death receptor. In this study, we adapted the Cell-SELEX technique by using the HCC cell line in targeting the Fas death receptor. By using the Cell-SELEX technique, identification of aptamer for targeted receptor was performed in its natural environment where the target was in its native conformation and in the same environment. In that way, the aptamer development is one step closer to the clinical translation^{162,163}. The HepG2 cell line was used as the positive cell because it expressed more

than 50% of the Fas death receptor. HUH7 cell line was used as the negative cell because it only expressed 1% of the Fas death receptor.

Since Cell-SELEX uses living cells, good cell culture maintenance is very important because dead cells due to overgrowth will cause an alteration in cell morphology and protein expression. Dead cells are detrimental to the success of the selection, but in this study, we considered them to be insignificant when using the adherent monolayer cells¹⁶⁴ HepG2 and Huh7. Moreover, dead cells were released in the medium and only living cells were detached in the positive selection.

Another thing important in the SELEX process is the retrieval of the sequences binding to target cells. We performed this by heating the cell-DNA complex at 95°C. The elevated temperature causes cell surface protein denaturation leading to the disruption of the DNA and protein interaction¹⁶². All the selected DNA pools of each round of selection were used for negative selection. Negative selections were performed in every cycle of Cell-SELEX after the second round of selection to filter out DNA sequences that might bind to the molecules existing on the surface of the target. It is important to eliminate unwanted DNA sequences that might contaminate the aptamer target pools.

PCR amplification is the main part of the process to enhance the success of the selection. The optimization of the annealing temperature and the concentration of all PCR reagents, including primers, were crucial. In this study, the optimal annealing temperature suitable for the DNA pool is 57°C and the numbers of annealing cycles were different in each round of selections based on the gel electrophoresis results that were performed to assess the PCR amplification efficiency.

The separation of the sense single-stranded DNA (ssDNA) and biotinylated antisense ssDNA was performed efficiently by affinity purification with streptavidin-coated magnetic beads. The obtained ssDNA pool was used for the round of selection. The last crucial step was screening and characterizing the DNA by cloning and sequencing.

The sequencing data was analyzed by using NCBI's BLAST and Bioedit program to select the candidate of the aptamer. The sequence that frequently appeared with high percentages

(around 90%) was selected as the aptamer candidate. From 40 clones, the putative aptamer candidate was labeled as RBL1. There was 3 predicted structure of RBL1 aptamer that was constructed by using *mfold* program. However, further analysis would be needed to confirm the result.

For conclusion, developing an oligonucleotide aptamer is hard and laborious work with a long experiment process. Nevertheless, the process was needed to achieve the potent aptamer that can specifically work in real condition. Further study must be done to test the efficiency and specificity of the aptamers for the future advantage

Chapter 5

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5. REFERENCES

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LIST OF PUBLICATIONS OBTAINED DURING PHD STUDY

Type	Year	Title	Authors	Journal	Conference	Volume (issue): page	DOI	ISSN
Article	2016	The role of gene polymorphisms of glutamate-cysteine ligase catalytic (GCLC) enzyme against antioxidants and oxidative stress status of Individual who had contacted infectious tuberculosis	Massi, M. Nasrum., Rafiah, S., Ladju, Rusdina Bte., Nurdin, G. M., Zulkifli, A. , Yuniastuti, A.	Malaysian Journal of Microbiology		12 (4): 322–326	http://dx.doi.org/10.21161/mjm.84516	2231-7538
Article	2017	Aptamer: a potential oligonucleotide nanomedicine againts hepatocellular carcinoma	Rusdina Bte Ladju, Devis Pascut, Muhammad N Massi, Caecilia Sukowati, Claudio Tiribelli	Oncotarget		9 (2): 2951–2961	10.18632/oncotarget.23359	1949–2553
Article	2018	Serum stem cell growth factor beta for the prediction of therapy response in hepatocellular carcinoma.	Sukowati, CHC., Patti, R., Pascut, D., Ladju, Rusdina Bte., Tarchi, P., Zanotta, N., Comar, C., Tiribelli, C., Croce, LS	BioMed Research International		2018: 6435482 (8 pages)	10.1155/2018/6435482	2314-6141

Abstract	2018	A new insight on the stem cell growth factor beta as a strong predictor of therapy response in hepatocellular carcinoma.	Sukowati, CHC., Patti, R., Pascut, D., Ladju, Rusdina Bte., Tarchi, P., Zanotta, N., Comar, C., Tiribelli, C., Croce, LS	Journal of Hepatology : The International Liver Congress 2018 Abstract Book	The International Liver Congress 2018	2018	https://doi.org/10.1016	0168-8278
Abstract	2018	PUMA and cellular death of FAS-mediated apoptosis in liver cancer	Ladju, Rusdina Bte., Massi, MN., Tiribelli, C., Sukowati, CHC	CROATIAN JOURNAL OF ONCOLOGY: Libri oncologici 2018	HDIR-5: "Translating Science to Medicine – Targets and Therapeutics"	XLVI		3008142
Abstract	2018	Induction of anti-FAS regulates PUMA and MYC: P53-dependent apoptosis pathway in hepatocellular carcinoma cells.	Ladju, Rusdina Bte., Tiribelli, C., Sukowati, CHC	ESMO OPEN. BMJ Journal	European Association for Cancer Research Congress 2018	2018	http://dx.doi.org/10.1136/esmoopen-2018-EACR25.133	

LIST OF INTERNATIONAL PRESENTATIONS CONDUCTED DURING PHD STUDY

Date	Activity type	Event description	Venue	notes
22-23/06/2017	workshop/congresses	29th Pezcoller Symposium: BUILDING NEW BRIDGES BETWEEN BASIC AND CANCER SCIENCE	Conference Hall, Fondazione CARITRO, via Calepina 1 38122 Trento - Italy	Poster Presentation
26-27/06/2017	workshop/congresses	WIN 2017 Symposium: Expediting Global Innovation in Precision Medicine	Hotel Novotel Paris Centre Tour Eiffel, Paris, France	Poster Presentation
15-Mar-18	lectures	Health Talk	Indonesian Embassy, Rome, Italy	Oral presentation
30-3/06-07/2018	workshop/congresses	25th Biennial Congress of the European Association for Cancer Research	Amsterdam RAI Exhibition and Convention Centre, Amsterdam, Netherland	Poster Presentation
14-16/02/2019	workshop/congresses	Hepatocellular Carcinoma Summit 2019	Lisbon Congress Centre, Lisbon, Portugal	E-Poster Presentation
