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EFFECT OF DOXORUBICIN AND PACLITAXEL ON THE SELECTIVE ONCOGENES EXPRESSION LEVEL OF HEPATOCELLULAR CARCINOMA RAS/RAF/MEK/ERK PATHWAY IN HUH-7 CELL LINE

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ABSTRACT

Hepatocellular carcinoma (HCC) is a serious health issue, and its prevalence is rapidly growing throughout the world. The expression in genes of cell signaling pathways has been correlated with HCC. This study explores the effect of doxorubicin and paclitaxel in preventing HCC. The effect of these drugs was evaluated on selective proteins in the hepatoma cell line. The Huh-7 cell line was cultured in Dulbecco's Modified Eagles Medium and treated with doses of doxorubicin and paclitaxel. The effect of these drugs on selected genes was evaluated through changes in mRNA level. The up and downregulation was determined by quantifying mRNA levels after cDNA synthesis and qPCR analysis. This study demonstrated that the doxorubicin promoted p53-DNA binding, which causes cells to undergo apoptosis. The paclitaxel drug arrests the cell cycle to increase the apoptosis rate by activating or phosphorylating the apoptotic proteins and hindering the antiapoptotic proteins. The CT values of the RT-PCR presented that the relative expression of selective oncogenes and growth factors involved in RAS/RAF pathway were downregulated upon the treatment of anti-cancerous doxorubicin and paclitaxel drug.

***Keywords*:** Hepatocellular carcinoma; RAS/RAF pathway; Oncogenes; Gene expression; Apoptosis

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1. INTRODUCTION

Hepatocellular carcinoma is a liver cancer that arises from hepatocytes and accounts for 80% of all liver cancer cases. Infectious agents and biomolecules sometimes overburden the liver, causing pathological conditions like hepatocellular carcinoma (HCC). Hepatitis viruses, drugs, and unwanted material in food items may cause overexpression or suppression of genes controlling the activities of hepatocytes, which may lead to fibrosis of the liver and cancer. HCC has experienced a rise in both incidence and death because of alterations in the social environment and lifestyle. This presents a serious risk to the well-being and health of individuals. HCC is a complex and common type of liver cancer that substantially threatens global health (Abdalla 2023; Shen et al. 2023). The high frequency of chronic hepatitis infection, resulting in 85–90% of cases of primary liver cancer, is the main cause of HCC (Zhou et al. 2018; Zou et al. 2022). Globally, HCC appears as the second most common cause of cancer-related fatalities, and its incidence is anticipated to increasing till 2030, with predictions of more than 1 million deaths from liver cancer (Zhou et al. 2018).

HCC is rising rapidly in developing countries due to the high frequency of cirrhosis from multiple reasons like nonalcoholic steatohepatitis (NASH), the higher survival ratio of cirrhotic patients, and the increasing chance of HCV infection and obesity (Ioannou 2021). NAFLD and NASH contribute to liver inflammation, oxidative stress, and fibrosis, making a microenvironment conducive to tumorigenesis (Mushtaq et al. 2024). Patients with cirrhosis and persistent HBV or HCV infections, in particular have a much greater risk of developing HCC than healthy individuals (Almeid et al. 2021; Kanwal et al. 2022). Chronic HBV/HCV infections are linked to persistent liver damage and inflammation, which increase the risk of genetic mutations and oncogene activation (Tian et al. 2022).

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Oncogenes to promote uncontrolled cell division and growth, are essential for the initiation and development of HCC (Zarlashat et al. 2024a,b).

HCC relies on the RAS/MAPK pathway, among others and dysregulation of this pathway often causes tumor development, invasion, and drug resistance. HCC is difficult to treat due to its resistance to standard treatments (Mushtaq et al. 2024). The dysregulation of the RAS/MAPK pathway is being extensively studied as biomarkers of the carcinogenic process that contribute to uncontrolled cell survival, proliferation, differentiation, and apoptosis (Yang and Liu 2017; Akula et al. 2019; Moon and Ro 2021). HCC cells actively manipulate the immune microenvironment and alter the cell physiology to influence the tumor's response to therapy (Donne and Lujambio 2022). Frequent alterations in cell signaling pathways are observed in HCC, resulting in reduced signaling cascades that contribute to the pathogenesis of the disease. Aberrant signaling pathways i.e., the dysregulated PI3K/AKT, Wnt/β-catenin, and MAPK/ERK cascades, often lead to elevated cell survival, enhanced proliferation, and resistance to apoptosis, thereby driving the progression of HCC (Garcia-Lezana et al. 2021; Fu et al. 2022).

Molecular mechanisms for the development of HCC are vital to developing effective therapeutic strategies (Ming et al. 2021). Treating HCC cells with a targeted inhibitor of the RAS/RAF pathway has been shown to significantly suppress tumor growth and induce apoptosis, highlighting the potential therapeutic value of targeting this pathway in HCC treatment (Samatar and Poulikakos 2014). Liver transplantation has become one of the top-notch treatments for HCC as it eliminates both the tumor and the underlying liver disease (Melloul et al. 2012). Complete resection of the tumor is the chosen treatment option for HCC, as it not only removes the tumor nevertheless also addresses the cirrhotic liver, a significant risk factor for HCC. However, if that is not feasible, there are alternative treatment options based on the stage, patient's condition, and available resources (Dimitroulis et al. 2017). Treatment of HCC poses significant challenges resulting from the few available therapeutic choices, the risk of tumor recurrence, and the potential for liver function impairment. The choice of therapy also introduces varying side effects that can impact the patient's overall well-being (Marin et al. 2021).

Conventional drugs such as chemotherapy and radiation often show low efficacy in HCC cases, supporting the need for searching novel and more potent therapeutic approaches. HCC resists conventional therapy, increasing the need for new therapies to improve patient outcomes and survival. Doxorubicin and paclitaxel are two widely recognized chemotherapy drugs that have shown great efficacy in treating several cancers. Their widely reported success in treating cancers promotes investigation into their potential effect on oncogenes and growth factors associated with the MAPK pathway to treat HCC. The study carefully selects nine particular genes based on their known involvement in the RAS/RAF pathway, an important protein in the progression of HCC. The main purpose of this study was to analyze the effect of doxorubicin and paclitaxel on the expression levels of distinct oncogenes and growth factors within the RAS/RAF pathway using the Huh-7 cell line of HCC. Further, this study aimed to systematically analyze the complex molecular interactions of the RAS/RAF pathway and the possible therapeutic effects of these chemotherapeutic drugs, providing novel strategies for treating HCC.

2. MATERIALS AND METHODS

2.1. Maintenance of Cell Line

Human hepatoma cells (Huh-7.0 cells) were retrieved from the National Institute for Biotechnology and Genetic Engineering (NIBGE) Cell Culture Collection (NCCC). Huh-7 cells were grown on DMEM (Cat. No. 11965-092, Thermo Scientific, USA) supplemented with heat-inactivated Fetal Bovine Serum (FBS, Gibco Life Science Technologies, USA), 1% Non-essential amino acids (NEAAs) and antibiotics (penicillin 100 units/mL, streptomycin 100units/mL) at 37ºC in 95% humified, 5% CO2 incubator. Huh-7 cells were maintained in 25cm^2 cell culture flasks via serial passaging: splitting monolayer with trypsin treatment every 3-4 days into a 1:3 ratio.

2.2. Doxorubicin and Paclitaxel Treatment

Doxorubicin and paclitaxel drugs were purchased from Sigma-Aldrich, USA. Dilutions for each drug (1nM, 10nM, 100nM, 250nM, 500nM, 1µM, 10µM, 50µM, 100µM) were prepared and treatments were given accordingly.

2.3. Estimation of 50% Growth Inhibitory Concentration and Cell Viability by MTT Assay

Growth inhibition (GI) was measured with a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, 96-well plates were seeded with Huh-7 cells (35,000cells/well), grown up to >95% confluency and treated with doxorubicin and paclitaxel drugs using prepared dilutions. After 72 hours of treatment, cells were subject to MTT assay. For MTT assay, 10µL of MTT agent (0.5mg/mL) was added in each well and incubated for 4 hours. The resulting stain was dissolved by adding 100µL of DMSO in each and shaking for 5 minutes. Finally, the absorbance was measured at 570nm in a multi-mode microplate reader (BioTek Instruments,

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USA). Cell viability was calculated by using % [100 × (sample abs/ control abs)] and GI was calculated by using GraphPad Prism (V. 10.3.1).

2.4. Gene Expression Analysis

2.4.1. RNA Isolation and cDNA Synthesis: For RNA extraction, Cell culture flasks were treated for 72 hours with respective drugs. RNA extraction from Huh-7 cells was performed with the TRIZOL method. In brief, TRIzol® reagent (1.0mL) was added in each sample and incubated for 10min to dissociate the nucleoprotein complexes. The chloroform 0.2mL per 1mL of TRIzol® reagent was added in each sample and centrifuged for 15min at 12,000×g. The combination was divided into an interphase, a lower red phenol-chloroform, and an upper colorless phase. The upper phase was transferred carefully without disturbing the interphase into a new tube, and each sample was then treated with 500µL of isopropyl for the precipitation of RNA. After centrifugation at 12,000×g for 10min, the RNA pellet was washed with 1mL of ethanol (75%), and the RNA pellet was air-dried and dissolved in 200µL of ultra-pure water. To remove DNA contamination, RNA was subjected to DNA digestion with Ambion® DNA-freeTM DNase Treatment and Removal Reagents kit (Cat No. AM 1906) according to manufacturer's guidelines. Further, RNA was purified from the digestion reaction by using FavorPrep™ Total RNA Isolation Kit (Cat No. FATRS 100, FAVORGEN Biotech Corporation, Taiwan) according to the manufacturer's guidelines. The purity of extracted RNA was determined by using a Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA). Finally, cDNA was synthesized with a SuperScript III First-Strand cDNA synthesis kit (Cat No. 18080051, Life Technologies).

2.5. Criteria for Genes Selection

Genes involved in RAS/RAF/MEK/ERK Pathway and growth factors PDGFR-β, VEGF, and HGF were selected on the basis of their vitality to the HCC.

2.6. Optimizing Primer Selection to Analyze Gene Expression

Primer 3 Plus was employed for designing primers from gene-specific sequences and validating the primer, i.e., query cover and percent identity of primer with specific gene by NCBI Blast (Table 1).

Table 1: Gene-specific primer sets for the gene expression analysis through RT-PCR

Gene symbol	Full name of the gene	Exon number	Total exons	Forward primer sequence 5′ to 3′ Reverse primer sequence 5′ to 3′	Product size
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	4	9	ACCCAGAAGACTGTGGATGG TTCTAGACGGCAGGTCAGGT	201
HRAS	Harvey rat sarcoma	4	5	GGCAGGAGACCCTGTAGGAG GGGTCGTATTCGTCCACAA	150
BRAF	B-RAF proto-oncogene	3	19	AATCTCTGGGGAACGGAACT TTTGTTGGGCAGGAAGACTC	191
MAPK	Mitogen-activated protein kinase	5	9	CAGGACCTCATGGAAACAGA ATCACAGGTGGTGTTGAGCA	174
MET	MET proto-oncogene	3	21	CCCAGATTGTTTCCCATGTC ACACTGGCTGGGCTCTTCTA	241
VEGF	Vascular endothelial growth factor C	1	7	GTTTGGAGGGGCTGAACAT CTCACAGGAAACCGGACATC	157
PDGFR-β	Platelet-derived growth factor receptor beta	5	23	TGCTGTTGCTGTCTCTCCTG TGAGGTTGGTCAGTGTGAGC	225
HGF	Hepatocyte growth factor	5	18	TCGAGCTATCGGGGTAAAGA AGCGTACCTCTGGATTGCTT	100

2.7. qPCR Amplification of Selected Genes

The SYBR green qPCR was chosen to analyze gene expression in control and treated samples. It is essential to assess the expression of a reference gene to normalize RNA expression in RT-PCR. The qPCR reaction mixture was assembled with 12.5µL SYBR green PCR master mix (Maxima SYBR Green/ROX qPCR Master Mix (2X)) along with forward 1µL and reverse primer 1µL, distilled water 8.5µL, and cDNA 2µL to make 25µL of total volume. The reaction was done by the iQTM5 Multi-color Real-Time PCR Detection System (BioRad). The thermal cycle was set up as follows: two minutes of initial denaturation at 95°C, fifteen seconds of denaturation at 95°C, forty-five seconds of annealing at 56–60°C, and one minute of extension at 72°C. Using the ΔΔCT technique, the fold induction was determined.

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3. RESULTS

3.1. Determination of MTT Assay

3.1.1. Doxorubicin Toxicity Analysis: Huh-7 cell line has been used to analyze the expression of selected oncogenes. The concentration in which over 80 percent of cells may survive was considered to be the non-toxic concentration. Results showed that doxorubicin at a dose of 5µL with 495µL of media from 10µM drug dilution was not toxic to Huh-7 cells. Therefore, the optimal 21.492µL of doxorubicin dose was selected for the treatment of Huh-7 cell line and used for gene expression analyses.

The effect of drugs on the Huh-7 cell line was checked to find any adverse effect on cells through MTT assay. Doxorubicin at 1nM showed maximum cell viability with minimum inhibition of growth and 7.12% of apoptosis. This may have a protective effect on cells and help maintain their survival. It was important to carefully determine the optimal concentration of the drug to achieve the desired therapeutic effect while minimizing any potential negative effect on the cell viability of nearby cells and tissues. This showed that doxorubicin changes the expression of certain viable genes in the cells. Increasing the concentration of doxorubicin to 100µM killed maximum number of cells with 96.28% mortality (Fig. 1, Fig. 2, and Table 2). Indeed at high concentrations, the drug may trigger an increased apoptosis which may have detrimental effects on nearby cells. It was significant to carefully consider the dosage and concentration of the drug to minimize potential harm to surrounding cells and tissues. The acceptable treatment was calculated to be 30µM for doxorubicin and all the subsequent experiments were conducted at this dose.

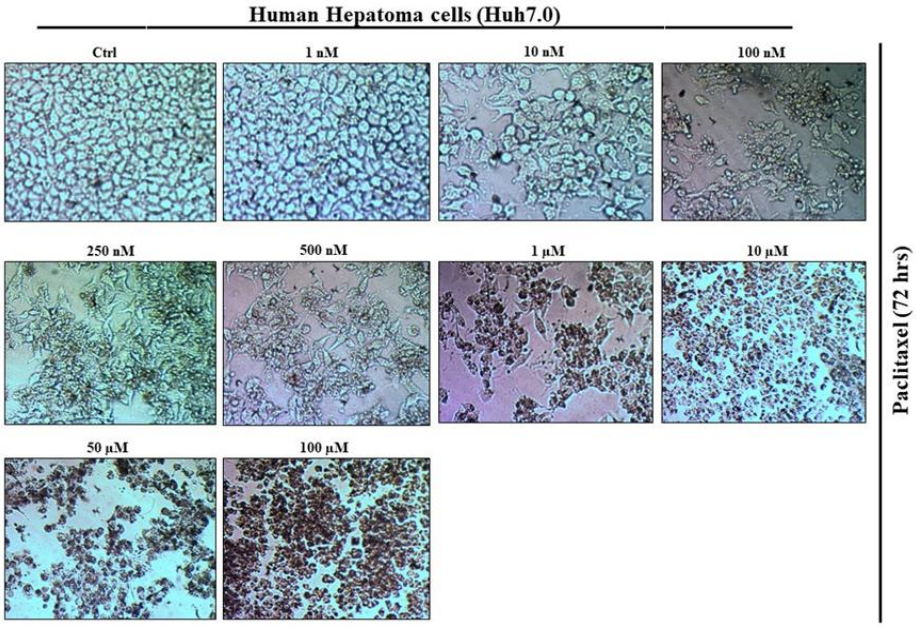

Fig. 1: A 10x inverted microscope image of doxorubicin treated Huh-7 cell line at different concentrations. It showed that these concentrations of doxorubicin were toxic and started apoptosis of Huh-7 cells. The rate of apoptosis at varying drug concentrations was analyzed by MTT assay after 72 hours of drugs treatment.

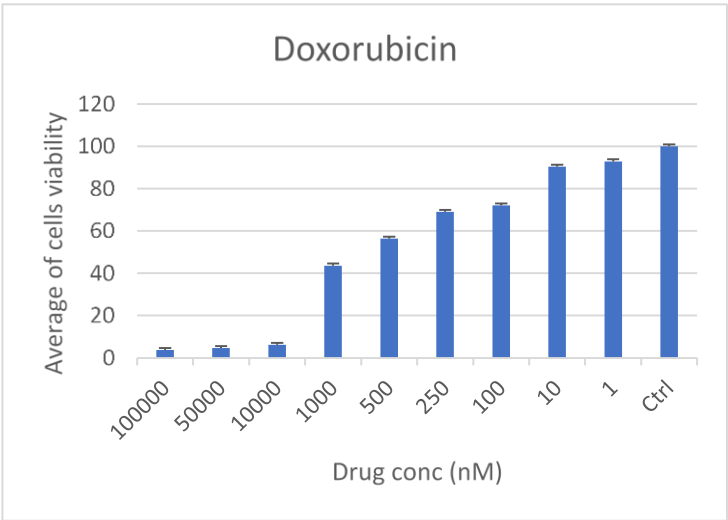

Fig. 2: The average of viable cells in different concentrations of doxorubicin drug on Huh-7 cell line.

Citation: Zarlashat Y, Ambreen A, Zafar MZ, Mushtaq H, Munir B, Mujahid M and Ghaffar A, 2024. Effect of doxorubicin and paclitaxel on the selective oncogenes expression level of hepatocellular carcinoma RAS/RAF/MEK/ERK pathway in Huh-7 cell line. Agrobiological Records 18: 1-11. https://doi.org/10.47278/journal.abr/2024.032

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Table 2: The different concentrations of doxorubicin showed the active cells, average number of active cells, and standard deviation to determine the toxicity of a drug.

Conc. (nM)	Doxorubicin			Cell viability 1	Cell viability 2	Cell viability 3	Mean+SD
100000	0.122	0.13	0.112	3.919049	4.145408	3.114572	3.726343+0.541763
50000	0.147	0.151	0.152	4.722133	4.815051	4.226919	4.588034±0.316167
10000	0.209	0.214	0.176	6.713781	6.82398	4.894327	6.144029±1.083675
1000	1.384	1.337	1.569	44.45872	42.63393	43.63181	43.57482±0.91373
500	1.688	1.863	1.99	54.22422	59.40689	55.33927	56.32346±2.727908
250	2.511	2.043	2.2	80.66174	65.14668	61.17909	68.99584±10.29589
100	2.537	2.22	2.301	81.49695	70.79082	63.98776	72.09184±8.826799
10	2.613	3.014	3.274	83.93832	96.10969	91.04561	90.36454±6.114201
1	2.652	3.074	3.432	85.19113	98.02296	95.43938	92.88449±6.786716
Ctrl	3.113	3.136	3.596	100	100	100	0

3.1.2. Paclitaxel Toxicity Analysis: Huh-7 cell line was employed to evaluate selected oncogenes expression involved in developing HCC. The concentration of paclitaxel in which 80% of cells may survive was said to be the non-toxic concentration. Results showed that paclitaxel at a dose of 5µL from 1µM of drug dilution was not toxic to Huh-7 cell line. Therefore, the optimal doxorubicin dose of 30µL for gene expression analyses.

The paclitaxel effect on the Huh-7 cell line was calculated using the MTT assay to identify the detrimental effects on cells. It was found that paclitaxel at a concentration of 1nM exhibited the highest cell viability, resulting in minimal growth inhibition and a 3.36% occurrence of apoptosis. This has the potential to protect cells and enhance the rate of survival. Consequently, it was of utmost importance to determine the most effective drug concentration that would produce the desired therapeutic benefits while simultaneously minimizing the risk of adverse effects on neighboring cells and tissues. This showed that paclitaxel induces changes in the expression of viable genes within cells. Increasing the paclitaxel concentration to 100µM resulted in the highest level of cell death, affecting 97.32% of the cell line (Fig. 3, Fig. 4, and Table 3). Furthermore, the drug may increase apoptosis at high concentrations, which may have detrimental effects on nearby cells. It was essential to carefully select the drug concentration to reduce the damage to nearby cells and tissues. The suitable dose of paclitaxel was determined to be 30µM, and all subsequent experiments were carried out at this dose.

3.2. Data Analysis of Real Time PCR

The data of RT-PCR was obtained as threshold cycle (CT) values and target genes expression compared to a GADPH expression. The relative expression level of the targeted genes were determined by CT 2^-(ΔΔCT values) (Fig. 5).

Table 3: The analysis of toxicity of paclitaxel to Huh-7 cell line by revealing active cells, average number of active cells upon drug treatment, and standard deviation.

Conc. (nM)	Paclitaxel			Cell viability 1	CV 2	CV 3	Mean±SD
100000	0.08	0.085	0.088	2.31615518	2.88919103	2.86365116	2.68966579±0.32372164
50000	0.094	0.097	0.09	2.72148234	3.29707682	2.92873414	2.9824311±0.29153006
10000	0.695	0.831	0.521	20.1215981	28.2460911	16.9541165	21.7739352±5.82450307
1000	1.838	1.743	1.662	53.2136653	59.2454113	54.083957	55.5143445±3.26036749
500	2.06	1.893	1.74	59.6409959	64.3439837	56.6221933	60.202391±3.891386
250	2.061	2.239	1.783	59.6699479	76.1046907	58.0214774	64.5987053±9.9985068
100	2.384	2.247	2.209	69.0214244	76.3766145	71.8841523	72.4273971±3.70756545
10	2.898	2.521	2.801	83.9027215	85.6900068	91.1487146	86.9138143±3.77483545
1	3.432	2.789	2.943	99.3630573	94.7994562	95.7696062	96.6440399±2.40418169
Ctrl	3.454	2.942	3.073	100	100	100	0

This study assessed how doxorubicin and paclitaxel affect selective oncogenes and growth factors involved in the RAS/RAF pathway responsible for HCC. Doxorubicin and paclitaxel-treated samples showed an altered level of gene expression. The gene expression of HRAS was decreased by the doxorubicin and paclitaxel treatment. This indicated that doxorubicin and paclitaxel have a strong effect on downregulating the twofold activity of this gene. Doxorubicin treatment resulted in a 0.223243 2^-(ΔΔCT) value decrease in HRAS gene expression, while paclitaxel treatment showed a 0.130449 2^-(ΔΔCT) value decrease the gene expression. Doxorubicin and paclitaxel also decreased the BRAF gene expression. This gene was highly expressed in the control sample of hepatoma cell lines. The 2^-(ΔΔCT) values described that BRAF was comparatively half expressed. Doxorubicin and paclitaxel treatment of the BRAF gene revealed the 2^-(ΔΔCT) values 0.442884 and 0.724471 hence indicating that the gene was downregulated in samples that were treated with doxorubicin and paclitaxel.

Citation: Zarlashat Y, Ambreen A, Zafar MZ, Mushtaq H, Munir B, Mujahid M and Ghaffar A, 2024. Effect of doxorubicin and paclitaxel on the selective oncogenes expression level of hepatocellular carcinoma RAS/RAF/MEK/ERK pathway in Huh-7 cell line. Agrobiological Records 18: 1-11. https://doi.org/10.47278/journal.abr/2024.032

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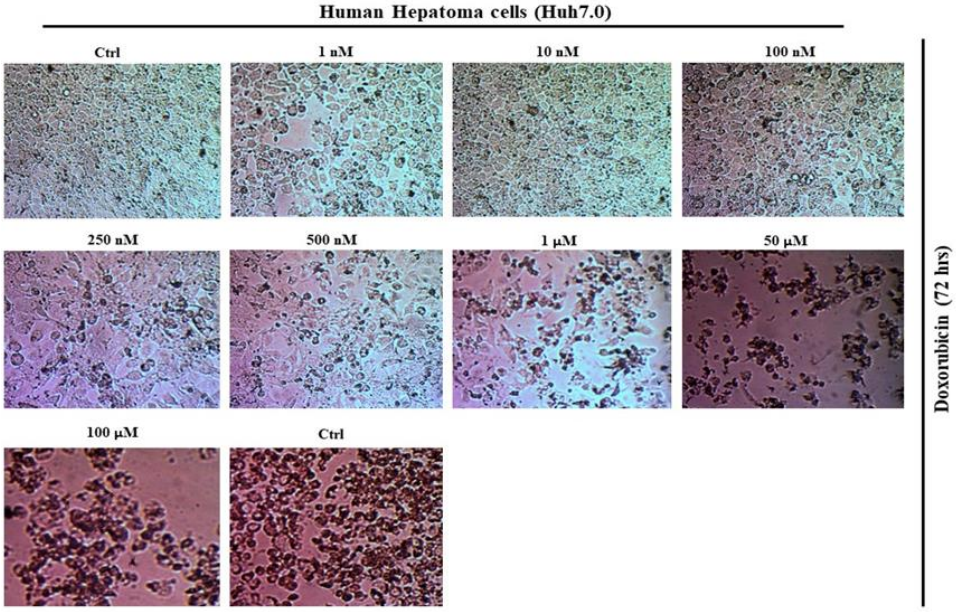

Fig. 3: A 10x inverted microscope images of paclitaxel treated Huh-7 cell line at different concentrations. It showed that high concentrations of paclitaxel were toxic to Huh-7 cells and low concentrations mediated the lesser rate of apoptosis.

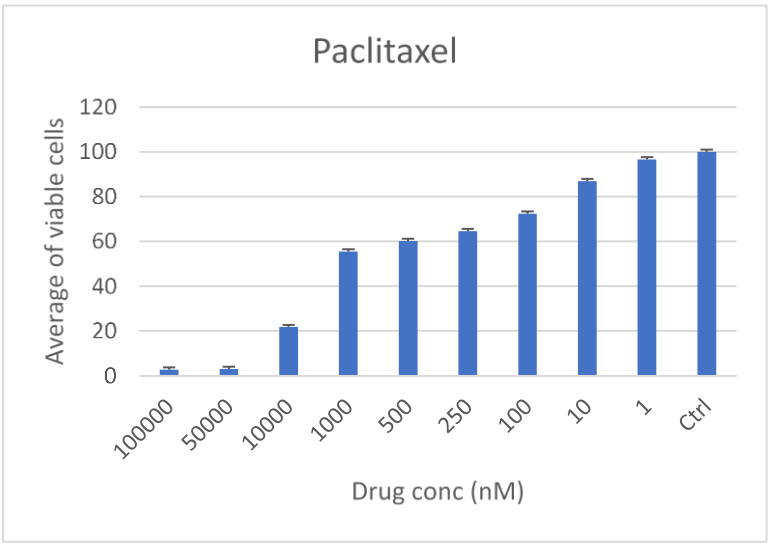

Fig. 4: The average of cells viability in different concentrations of paclitaxel drug on Huh-7 cell line.

The MAPK gene was downregulated by both doxorubicin and paclitaxel but less than HRAS and BRAF. The gene expression value was 0.765779 by doxorubicin and 0.692555 by paclitaxel. Doxorubicin and paclitaxel have a slight effect on gene expression of MAPK, suggesting that these drugs were not strong inducers or inhibitors of MAPK gene. The relative expression of MET in the Huh-7 cells with doxorubicin and paclitaxel was reduced by twofold but less than HRAS. The 2^(-ΔΔCT) value of the gene was 0.286181 upon doxorubicin treatment and 0.276433 paclitaxel treatment which indicated that doxorubicin and paclitaxel drug were sufficient to downregulate this oncogene.

Doxorubicin and paclitaxel downregulated the PDGFR-β and VEGF gene leading to decreased proliferation. Doxorubicin and paclitaxel seem to have a complex and equal effect on gene expression, both downregulating growth factors and receptor tyrosine kinase genes. The 2^-(ΔΔCT) value of the PDGFR-β was 0.786783 with doxorubicin treatment and 0.401312 with paclitaxel treatment. The VEGF gene exhibited a 2^-(ΔΔCT) value of 0.850308 with doxorubicin treatment and 0.400535 with paclitaxel treatment. These values indicated that both doxorubicin and paclitaxel effectively downregulate these genes. The 2^-(ΔΔCT) value revealed lower expression of PDGFR-β and VEGF in Huh-7 cells. This value indicated that doxorubicin and paclitaxel have the potency to downregulate this gene.

The HGF expression was also found to be decreased. It was fascinating to observe how doxorubicin and paclitaxel may significantly affect gene expression in Huh-7 cell line. The 0.102593 2^-(ΔΔCT) of HGF by

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doxorubicin and 0.240649 2^-(ΔΔCT) by paclitaxel treatment, have lowered the level of gene expression (Fig. 5, Table 4). Doxorubicin and paclitaxel were a strong HGF gene inhibitor as shown by the drug-treated sample's 2^-(ΔΔCT) value. The decreased expression of growth factors in Huh-7 cell line by doxorubicin and paclitaxel doesn't necessarily imply a direct link with HCC.

Table 4: The mean CT value and the ΔCT value for housekeeping gene and targeted gene were presented. The 2^-(ΔΔCT values) was the convenient way to evaluate relative changes in gene expression of target genes

Gene targeted									
		GAPDH	HRAS	BRAF	MAPK	MET	VEGF	PDGFR-β	HGF
Mean C_T	Control	32.34	35.39	36.68	35.51	35.04	35.18	33.57	35.33
	Doxorubicin	28.72	33.93	34.24	32.27	33.22	31.90	30.18	35.00
	Paclitaxel	25.81	31.80	30.61	29.50	30.36	29.96	28.44	30.85
ΔC_T	Control	-	3.05	4.34	3.16	2.69	2.83	1.22	2.99
	Doxorubicin	-	5.21	5.52	3.55	4.50	3.18	1.46	6.28
	Paclitaxel	-	5.99	4.81	3.70	4.55	4.16	2.64	5.05
2^-(ΔΔCT values)	Control	-	1	1	1	1	1	1	1
	Doxorubicin	-	0.223	0.443	0.766	0.286	0.787	0.850	0.103
	Paclitaxel	-	0.130	0.724	0.693	0.276	0.401	0.376	0.241

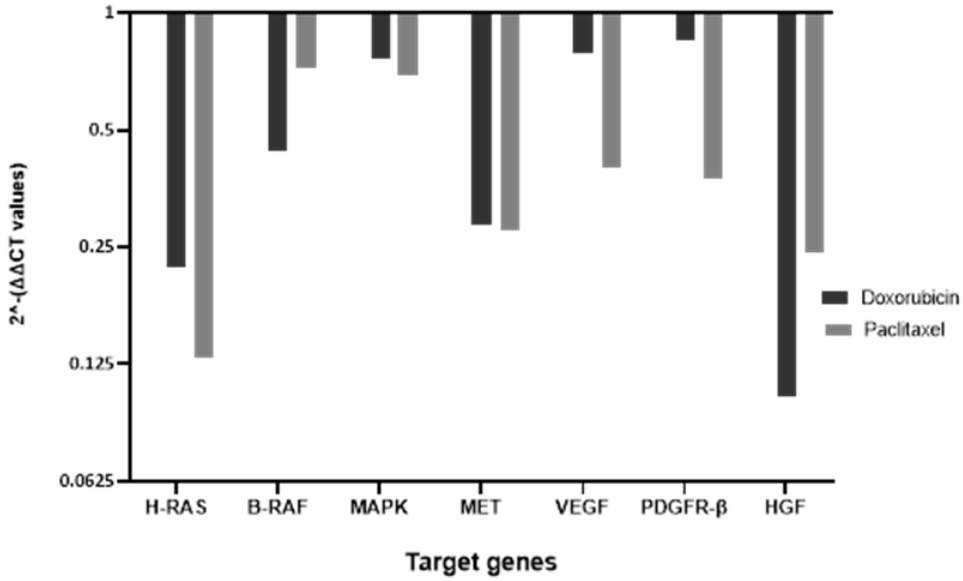

Fig. 5: The expression fold change of different oncogenes and growth factors involved in RAS/RAF pathway in Huh-7 cell line after treated with anticancerous drugs doxorubicin and paclitaxel. The doxorubicin 21.492µL and paclitaxel 30µL were used to check the expression fold change of nine genes. 2^-(ΔΔCT) was shown that this concentration of drugs downregulated the gene expression of selected oncogenes and growth factors.

4. DISCUSSION

This study revealed that the chemotherapy drugs doxorubicin and paclitaxel have significant effects on the RAS/RAF pathway, leading to the development and proliferation of HCC cells. These drugs which are often used in chemotherapy and have varied effects on this pathway, suggesting their potential for treating HCC. It was found that doxorubicin and paclitaxel may selectively regulate the oncogene expression level within the RAS/RAF/MEK pathway.

Doxorubicin, a chemotherapeutic drug in cancer treatment, is used to cause apoptosis in HCC cell lines (Liu et al. 2019). The pharmacological molecule specifically inhibits the repair of the DNA double helix, interrupting the replication process once the topoisomerase II has destroyed the DNA in the replication process (Buzun et al. 2020). Additionally, it has been hypothesized that doxorubicin promotes p53-DNA binding, which causes cells to undergo apoptosis (Cai et al. 2022). The findings indicated that doxorubicin induces apoptosis in cells by upregulating pro-apoptotic factors like Bax, caspase-3, and caspase-8 (Jiang et al. 2020).

Paclitaxel stabilizes the structure of microtubules through assembly and disassembly to prevent cell division in the G2/M phase (Leung and Cassimeris 2019). Mitotic checkpoint activation prevents continuous mitosis, either causing apoptosis or a return to the G-phase, and paclitaxel-induced cell death involves Bcl-2 phosphorylation, diminishing its antiapoptotic activity (Kadry et al. 2020). The paclitaxel promotes apoptosis in cancer cells by triggering the BRCA1 and p38 signaling pathways through the stimulation of tumor suppressor genes PTEN and p53 (Zhao et al. 2022).

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Doxorubicin and paclitaxel therapy significantly downregulated the RAS oncogene. The H-RAS gene demonstrated 0.223 ΔΔCT values with doxorubicin treatment and 0.130 with paclitaxel treatment. The fold change showed the gene was downregulated in both doxorubicin and paclitaxel-treated samples. H-RAS-mutated C643 cell lines exhibited enhanced cytotoxicity with doxorubicin, paclitaxel, and tipifarnib, as indicated by their significantly lower IC50 values (Lopes-Ventura et al. 2019). In cancer cells, doxorubicin causes cell cycle arrest and downregulates genes such as HRAS in the cell cycle to prevent further cell division (Mebratu and Tesfaigzi 2009). Paclitaxel downregulates the HRAS gene because it interferes with microtubule dynamics in cancer cells, disrupting the normal cell division process and inhibiting the RAS/RAF signaling pathway (Eritja et al. 2017).

The level of expression of the B-RAF gene was decreased by the treated sample with either doxorubicin and paclitaxel treatment. The treated sample's CT value demonstrated that either doxorubicin or paclitaxel significantly inhibits the B-RAF gene. The protein kinase RAF regulates apoptosis by phosphorylating proteins directly via MEK/ERK activation (Ullah et al. 2022). The combination of miR34a and doxorubicin was observed to suppress the RAS/RAF pathway therefore slowing down the tumor growth (Yang et al. 2021). Doxorubicin may downregulate the BRAF gene in Huh-7 cells due to its cytotoxic effects, which can contribute to DNA damage and activation of cellular stress responses, thereby potentially causing alterations in gene expression, including BRAF downregulation (Li et al. 2021).

Doxorubicin and paclitaxel have inhibitory effects on gene expression of selected oncogenes and growth factors H-RAS, B-RAF, MAPK, VEGF, MET, PDGFR-β, and HGF for liver carcinoma. HCC exhibits increased RAF kinase expression, a serine/threonine kinase family member, which influences cell proliferation, differentiation, and division (Gnoni et al. 2019). The MAPK pathway is responsible for controlling many biochemical signals related to several pathways to carry out the functions of genes (Raja et al. 2017). Dysregulation of MAPK cascades to regulate diverse biological processes through ERK phosphorylation and nuclear target phosphorylation, which underlies the development of disorders including cancer, autoimmune diseases, diabetes, and developmental abnormalities, often involving the transcription of regulatory genes (Tikkanen and Paterson 2019). In this study, it was observed the use of doxorubicin decreased the MAPK gene expression by 0.766 ΔΔCT in hepatoma cell lines, while paclitaxel resulted in a reduction of 0.693 ΔΔCT specifically in the Huh-7 cells. The downregulation of the MAPK gene in Huh-7 cells by paclitaxel linked its ability to induce cellular stress, disrupt microtubule dynamics, and affect upstream signaling pathways (Krelle et al. 2013).

The MET gene, also known as c-MET, is associated with hepatocyte growth factor (HGF) as its primary ligand, functioning as a receptor for various growth factors to initiate diverse signaling pathways, ultimately regulating cellular proliferation (Fu et al. 2021). The treatment with doxorubicin (0.286 ΔΔCT) and paclitaxel (0.276 ΔΔCT) showed a modest decrease in the relative expression of the MET gene, suggesting the activity of c-MET gene is downregulated by these treatments. MiR-34b overexpression in cancerous cells inhibits MET oncogene expression. According to recent research, inhibiting MET expression regulates chemotherapy-linked apoptosis and paclitaxel sensitivity (Yanokura et al. 2020). In cancer, doxorubicin and paclitaxel downregulate the MET gene by disturbing signaling pathways and altering transcriptional regulation, thereby inhibiting its expression and possibly inhibiting tumor growth (Mirzaei et al. 2021).

The PDGFR protein is a cell surface receptor and is found to be overexpressed in many cancers (Pandey et al. 2023). This study indicated that treating hepatoma cell lines with doxorubicin reduced the expression of the PDGFR-β gene by 0.850 ΔΔCT, and paclitaxel reduced it by 0.376 ΔΔCT in the Huh-7 cell line. This suggested that lower expression of cellular growth factors reduces HCC development. The PDGFR inhibitor with doxorubicin not only inhibited cell growth but also increased apoptotic cell death more consistently compared to doxorubicin alone. This advised that the combination treatment could be a more effective approach to targeting cancer cells (Kim et al. 2021). The combination of STI571 and paclitaxel treatment not only inhibited phosphorylation of PDGFR in cancerous cells and cancer-related endothelial cells, but it also led to regulated apoptosis, lowered proliferation, and lesser microvessel in the tumors (Lev et al. 2005).

Doxorubicin and paclitaxel treatment resulted in a reduction in HGF expression in the Huh-7 cells. Paclitaxel has a ΔΔCT 0.241, and doxorubicin ΔΔCT 0.103 showed a decrease in HGF gene expression. Doxorubicin and paclitaxel reduced the gene expression of HGF in cancer by blocking the gene transcription of HGF and altering signaling pathways, which may inhibit tumor development (Subramaniam et al. 2013). VEGF, a critical component of angiogenesis, promotes the development of new blood vessels and tumor growth by stimulating chemotaxis and mitogenesis in healthy endothelial cells while also increasing vascular permeability (Geindreau et al. 2022). The treated hepatoma cell lines with doxorubicin reduced the expression of the VEGF gene by 0.787 ΔΔCT, and paclitaxel reduced it by 0.401 ΔΔCT in the Huh-7 cell line. The given results of RT-PCR demonstrated that doxorubicin decreased the VEGF mRNA expression by 46.84±3.72% (Sun et al. 2018). Paclitaxel not only reduces VEGF expression in both Met-1 cells in mice and cell culture but also suggests that this VEGF downregulation is an extra mechanism through which paclitaxel exerts its antiangiogenic effects

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(Kaumaya and Foy 2012). HGF is crucial for mesenchymal growth, cell invasion, and tumor metastasis, while VEGF primarily produced by vascular endothelial cells, promotes cancer development when expressed at high levels (Noubissi et al. 2022).

5. CONCLUSION

HCC is the most growing health challenge in the globe. The therapy of advanced HCC is now a very active topic of research since HCC remains one of the worst cancers with a low chance of survival. A thorough knowledge of the abnormally regulated proteins involved in RAS/RAF/MEK pathway leading to carcinogenesis. Hepatocarcinogenesis is a complex, multistep process characterized by genetic alterations to over-activate protooncogenes and deactivate tumor suppressor genes, subsequently leading to modifications in various signaling cascades. The RAS/RAF pathway is the most effective signaling pathway that controls HCC and apoptosis in cancer. This research highlights the anticancerous activity of doxorubicin and paclitaxel on genetic expression of oncogenes and growth factors to regulate the RAS/RAF pathway in HCC. The results revealed that doxorubicin and paclitaxel can increase apoptosis rate and downregulate these genes involved in RAS/RAF pathway according to the CT value. This suggests that these drugs may be used to inhibit hepatocellular carcinoma. Future research focused on combination treatments, personalized therapies, and translational applications to improve treatment outcomes on the anticancer activity in HCC.

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REFERENCES

Abdalla MMI, 2023. Serum resistin and the risk for hepatocellular carcinoma in diabetic patients. World Journal of Gastroenterology, 29(27): 4271. https://doi.org/10.3748/wjg.v29.i27.4271

Akula SM, Abrams SL, Steelman LS, Emma MR, Augello GCA, Azzolina A, Montalto G, Cervello M and McCubrey JA, 2019. RAS/RAF/MEK/ERK, PI3K/PTEN/AKT/mTORC1 and TP53 pathways and regulatory miRs as therapeutic targets in hepatocellular carcinoma. Expert Opinion on Therapeutic Targets, 23(11): 915-929. https://doi.org/10.1080/14728222.2019.1685501

Almeid PH, Matielo CE, Curvelo, LA, Rocco RA, Felga G, Della Guardia B and Boteon YL, 2021. Update on the management and treatment of viral hepatitis. World Journal of Gastroenterology, 27(23): 3249. https://doi.org/10.3748/wjg.v27.i23.3249

Buzun K, Bielawska A, Bielawski K and Gornowicz A, 2020. DNA topoisomerases as molecular targets for anticancer drugs. Journal of Enzyme Inhibition and Medicinal Chemistry, 35(1): 1781-1799. https://doi.org/10.1080/14756366.2020.1821676

Cai H, Tian P, Ju J, Wang T, Chen X, Wang K, Wang F, Yu X, Wang S and Wang Y, 2022. Long noncoding RNA NONMMUT015745 inhibits doxorubicin-mediated cardiomyocyte apoptosis by regulating Rab2A-p53 axis. Cell Death Discovery, 8(1): 364. https://doi.org/10.1038/s41420-022-01144-9

Dimitroulis D, Damaskos C, Valsami S, Davakis S, Garmpis N, Spartalis E, Athanasiou A, Moris D, Sakellariou S and Kykalos S, 2017. From diagnosis to treatment of hepatocellular carcinoma: An epidemic problem for both developed and developing world. World Journal of Gastroenterology, 23(29): 5282. https://doi.org/10.3748/wjg.v23.i29.5282

Donne R and Lujambio A, 2022. The liver cancer immune microenvironment: Therapeutic implications for hepatocellular carcinoma. Hepatology, 77(5): 1773-1796. https://doi.org/https://doi.org/10.1002/hep.32740

Eritja N, Yeramian A, Chen BJ, Llobet-Navas D, Ortega E, Colas E, Abal M, Dolcet X, Reventos J and Matias-Guiu X, 2017. Endometrial carcinoma: specific targeted pathways. Molecular Genetics of Endometrial Carcinoma, 149-207. https://doi.org/10.1007/978-3-319-43139-0_6

Fu J, Su X, Li Z, Deng L, Liu X, Feng X and Peng J, 2021. HGF/c-MET pathway in cancer: from molecular characterization to clinical evidence. Oncogene, 40(28): 4625-4651. https://doi.org/10.1038/s41388-021-01863-w

Fu D, Hu Z, Xu X, Dai X and Liu Z, 2022. Key signal transduction pathways and crosstalk in cancer: Biological and therapeutic opportunities. Translational Oncology, 26: 1015https://doi.org/10. https://doi.org/10.1016/j.tranon.2022.101510

Garcia-Lezana T, Lopez-Canovas JL and Villanueva A, 2021. Signaling pathways in hepatocellular carcinoma. Advances in Cancer Research, 149: 63-101. https://doi.org/https://doi.org/10.1016/bs.acr.2020.https://doi.org/10.002

Geindreau M, Bruchard M and Vegran F, 2022. Role of cytokines and chemokines in angiogenesis in a tumor context. Cancers, 14(10): 2446. https://doi.org/10.3390/cancers14102446

Gnoni A, Licchetta A, Memeo R, Argentiero A, Solimando A G, Longo V, Delcuratolo S and Brunetti O, 2019. Role of BRAF in hepatocellular carcinoma: a rationale for future targeted cancer therapies. Medicina, 55(12): 754. https://doi.org/10.3390/medicina55120754

ISSN: 2708-7182 (Print); ISSN: 2708-7190 (Online)
Open Access Journal

Ioannou GN, 2021. Epidemiology and risk-stratification of NAFLD-associated HCC. Journal of Hepatology, 75(6): 1476-1484. https://doi.org/10.1016/j.jhep.2021.08.012

Jiang M, Qi L, Li L and Li Y, 2020. The caspase-3/GSDME signal pathway as a switch between apoptosis and pyroptosis in cancer. Cell Death Discovery, 6(1): 112. https://doi.org/10.1038/s41420-020-00349-0

Kadry H, Noorani B and Cucullo L, 2020. A blood–brain barrier overview on structure, function, impairment, and biomarkers of integrity. Fluids and Barriers of the CNS, 17(1): 1-24. https://doi.org/10.1186/s12987-020-00230-3

Kanwal F, Khaderi S, Singal AG, Marrero JA, Loo N, Asrani SK, Amos CI, Thrift AP, Gu X and Luster M, 2022. Risk factors for HCC in contemporary cohorts of patients with cirrhosis. Hepatology. https://doi.org/10.1002/hep.32434

Kaumaya PT and Foy KC, 2012. Peptide vaccines and peptidomimetics targeting HER and VEGF proteins may offer a potentially new paradigm in cancer immunotherapy. Future Oncology, 8(8): 961-87. https://doi.org/10.2217/fon.12.95

Kim S, You D, Jeong Y, Yoon SY, Kim SA and Lee JE, 2021. Inhibition of platelet-derived growth factor C and their receptors additionally increases doxorubicin effects in triple-negative breast cancer cells. European Journal of Pharmacology, 895: 173868. https://doi.org/10.1016/j.ejphar.2021.173868

Krelle AC, Okoli AS and Mendz GL, 2013. Huh-7 human liver cancer cells: a model system to understand hepatocellular carcinoma and Therapy. https://doi.org/10.4236/jct.2013.42078

Leung JC and Cassimeris L, 2019. Reorganization of paclitaxel-stabilized microtubule arrays at mitotic entry: roles of depolymerizing kinesins and severing proteins. Cancer Biology & Therapy, 20(10): 1337-1347. https://doi.org/10.1080/15384047.2019.1638678

Lev DC, Kim SJ, Onn A, Stone V, Nam DH, Yazici S, Fidler IJ and Price JE, 2005. Inhibition of platelet-derived growth factor receptor signaling restricts the growth of human breast cancer in the bone of nude mice. Clinical Cancer Research, 11(1): 306-314. https://doi.org/10.1158/1078-0432.306.11.1

Li Y, Tang T, Lee HJ and Song K, 2021. Selective anti-cancer effects of plasma-activated medium and its high efficacy with cisplatin on hepatocellular carcinoma with cancer stem cell characteristics. International Journal of Molecular Sciences, 22(8): 3956. https://doi.org/10.3390/ijms22083956

Liu Y, Liu L, Zhou Y, Zhou P, Yan Q, Chen X, Ding S and Zhu F, 2019. CKLF1 enhances inflammation-mediated carcinogenesis and prevents doxorubicin-induced apoptosis via IL6/STAT3 signaling in HCC. Clinical Cancer Research, 25(13): 4141-4154. https://doi.org/10.1158/1078-0432.CCR-18-3510

Lopes-Ventura S, Pojo M, Matias A, Moura M, Marques I, Leite V and Cavaco B, 2019. The efficacy of HRAS and CDK4/6 inhibitors in anaplastic thyroid cancer cell lines. Journal of Endocrinological Investigation, 42: 527-540. https://doi.org/10.1007/s40618-018-0947-4

Marin JJ, Romero MR, Herraez E, Asensio M, Ortiz-Rivero S, Sanchez-Martin A, Fabris L and Briz O, 2021. Mechanisms of pharmacoresistance in hepatocellular carcinoma: New drugs but old problems. Seminars in Liver Disease, 42(1): 87-103. https://doi.org/10.1055/s-0041-1735631

Mebratu Y and Tesfaigzi, 2009. How ERK1/2 activation controls cell proliferation and cell death: Is subcellular localization the answer? Cell Cycle, 8(8): 1168-1175. https://doi.org/10.4161/cc.8.8.8147

Melloul E, Lesurtel M, Carr BI and Clavien PA, 2012. Developments in liver transplantation for hepatocellular carcinoma. Seminars in Oncology, 39(4): 510-21. https://doi.org/https://doi.org/10.1053/j.seminoncol.2012.05.008

Ming H, Li B, Zhou L, Goel A and Huang C, 2021. Long non-coding RNAs and cancer metastasis: Molecular basis and therapeutic implications. Biochimica et Biophysica Acta (BBA)-Reviews on Cancer, 1875(2): 188519. https://doi.org/10.1016/j.bbcan.2021.188519

Mirzaei S, Mahabady MK, Zabolian A, Abbaspour A, Fallahzadeh P, Noori M, Hashemi F, Hushmandi K, Daneshi S and Kumar AP, 2021. Small interfering RNA (siRNA) to target genes and molecular pathways in glioblastoma therapy: Current status with an emphasis on delivery systems. Life Sciences, 275: 119368. https://doi.org/10.1016/j.lfs.2021.119368

Moon H and Ro SW, 2021. MAPK/ERK signaling pathway in hepatocellular carcinoma. Cancers, 13(12): 3026. https://doi.org/10.3390/cancers13123026

Mushtaq H, Zarlashat Y, Ambreen A, Mujahid M, Kausar S and Shafqat D, 2024. Reviewing advances in understanding and targeting the MAPK signaling pathway in hepatocellular carcinoma progression and therapeutics. Agrobiological Records, 15: 103-116. https://doi.org/https://doi.org/10.47278/journal.abr/2024.004

Noubissi NGA, Geismann C, Arlt A, Hoogwater FJ, Nijkamp MW, Meyer NH and Bockhorn M, 2022. Role of epithelial-to-mesenchymal transition for the generation of circulating tumors cells and cancer cell dissemination. Cancers, 14(22): 5483. https://doi.org/10.3390/cancers14225483

Pandey P, Khan F, Upadhyay TK, Seungjoon M, Park MN and Kim B, 2023. New insights about the PDGF/PDGFR signaling pathway as a promising target to develop cancer therapeutic strategies. Biomedicine and Pharmacotherapy, 161: 114491. https://doi.org/10.1016/j.biopha.2023.114491

Raja V, Majeed U, Kang H, Andrabi KI and John R, 2017. Abiotic stress: Interplay between ROS, hormones and MAPKs. Environmental and Experimental Botany, 137: 142-157. https://doi.org/10.1016/j.envexpbot.2017.02.010

Samatar AA and Poulikakos PI, 2014. Targeting RAS–ERK signalling in cancer: promises and challenges. Nature Reviews Drug Discovery, 13(12): 928-942. https://doi.org/10.1038/nrd4281

Shen C, Jiang X, Li M and Luo Y, 2023. Hepatitis virus and hepatocellular carcinoma: Recent advances. Cancers, 15(2): 533. https://doi.org/10.3390/cancers15020533

Subramaniam A, Shanmugam MK, Perumal E, Li F, Nachiyappan A, Dai X, Swamy SN, Ahn KS, Kumar AP and Tan BK, 2013. Potential role of signal transducer and activator of transcription (STAT) 3 signaling pathway in inflammation, survival,

ISSN: 2708-7182 (Print); ISSN: 2708-7190 (Online)
Open Access Journal

proliferation and invasion of hepatocellular carcinoma. Biochimica et Biophysica Acta (BBA)-Reviews on Cancer, 1835(1): 46-60. https://doi.org/10.1016/j.bbcan.2012.https://doi.org/10.002

Sun Q, Wang X, Cui C, Li J and Wang Y, 2018. Doxorubicin and anti-VEGF siRNA co-delivery via nano-graphene oxide for enhanced cancer therapy in vitro and in vivo. International Journal of Nanomedicine, 3713-3728. https://doi.org/10.2147/IJN.S162939

Tian Z, Xu C, Yang P, Lin Z, Wu W, Zhang W, Ding J, Ding R, Zhang X and Dou K, 2022. Molecular pathogenesis: Connections between viral hepatitis-induced and non-alcoholic steatohepatitis-induced hepatocellular carcinoma. Frontiers in Immunology, 13: 984728. https://doi.org/10.3389/fimmu.2022.984728

Tikkanen R and Nikolic-Paterson DJ, 2019. Mitogen-activated protein kinases: Functions in signal transduction and human diseases. International Journal of Molecular Sciences, 20(19): 4844. https://doi.org/10.3390/ijms20194844

Ullah R, Yin Q, Snell AH and Wan L, 2022. RAF-MEK-ERK pathway in cancer evolution and treatment. Seminars in Cancer Biology, 85:123-154. https://doi.org/10.1016/j.semcancer.2021.05.010

Yang S and Liu G, 2017. Targeting the Ras/Raf/MEK/ERK pathway in hepatocellular carcinoma. Oncology Letters, 13(3): 1041-1047. https://doi.org/10.3892/ol.2017.5557

Yang X, Shang P, Yu B, Jin Q, Liao J, Wang L, Ji J and Guo X, 2021. Combination therapy with miR34a and doxorubicin synergistically inhibits Dox-resistant breast cancer progression via down-regulation of Snail through suppressing Notch/NF-κB and RAS/RAF/MEK/ERK signaling pathway. Acta Pharmaceutica Sinica B, 11(9): 2819-2834. https://doi.org/10.1016/j.apsb.2021.06.003

Yanokura M, Banno K and Aoki D, 2020. MicroRNA-34b expression enhances chemosensitivity of endometrial cancer cells to paclitaxel. International Journal of Oncology, 57(5): 1145-1156. https://doi.org/10.3892/ijo.2020.5127

Zarlashat Y, Abbas S and Ghaffar A, 2024a. Hepatocellular Carcinoma: Beyond the Border of Advanced Stage Therapy. Cancer, 16(11): 2034. https://doi.org/10.3390/cancers16112034

Zarlashat Y, Mushtaq H, Pham L, Abbas W and Sato K, 2024b. Advancements in Immunotherapeutic Treatments for Hepatocellular Carcinoma: Potential of Combination Therapies. International Journal of Molecular Sciences, 25(13), 6830. https://doi.org/10.3390/ijms25136830

Zhao S, Tang Y, Wang R and Najafi M, 2022. Mechanisms of cancer cell death induction by paclitaxel: an updated review. Apoptosis, 27(9-10): 647-667. https://doi.org/10.1007/s10495-022-01750-z

Zhou J, Sun HC, Wang Z, Cong WM, Wang JH, Zeng MS, Yang JM, Bie P, Liu LX and Wen TF, 2018. Guidelines for diagnosis and treatment of primary liver cancer in China (2017 Edition). Liver Cancer, 7(3): 235-260. https://doi.org/10.1159/000488035

Zou H, Li M, Lei Q, Luo Z, Xue Y, Yao D, Lai Y, Ung COL and Hu H, 2022. Economic burden and quality of life of hepatocellular carcinoma in greater China: A systematic review. Frontiers in Public Health, 10: 801981. https://doi.org/10.3389/fpubh.2022.801981