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Hesa-A Effects on Cell Cycle Signaling in Esophageal Carcinoma Cell Line

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ABSTRACT

BACKGROUND

Hesa-A is a natural compound with anticancer properties. The exact mechanism of its action in esophageal cancer is not clear, yet. The aim of this study was to evaluate the cell toxicity effect of Hesa-A on the esophageal carcinoma cell lines, KYSE-30, and cell cycle genes expression.

METHODS

In this study, we tested cell toxicity with MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay and flow cytometry to evaluate the cell cycle arrest. Real time polymerase chain reaction was used to assess the expression of P53, P16, P21, cyclin D1, and cyclin B1 genes.

RESULTS

Our results showed that Hesa-A is effective in the expression of cell cycling check point proteins. Hesa-A induced an arrest in G2 phase of esophageal cell cycle. The levels of P53 (>13 times), P21 (>21 times), P16, cyclin B1, and cyclin D1 genes were increased 48 hours after Hesa-A treatment.

CONCLUSION

P21 and P16 expression were the potential mechanisms for G2 arrest of KYSE-30 esophageal cancer cell line by Hesa-A.

KEYWORDS

Hesa-A; Esophageal cancer; Flow cytometry; Real Time PCR; P53 gene; P16 gene; P21 gene; Cyclin D1 gene; Cyclin B1 gene.

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INTRODUCTION

Esophageal squamous cell carcinoma occurs in 50 of 100,000 individuals each year in the northeast Iran (Golestan region), which is one of the highest rates in the world.[1] Various genes are contributed in the suppression of cancer cells, but sometimes their expression is changed, which leads to the cancer development.[2]

P53 (from P53 gene) is an anti-oncogene nuclear protein, which has critical role in the apoptosis and cell cycle.[3-5] Its function accomplishes through activation of downstream genes like P21.[6] P21 and P16 proteins are cyclin dependant kinas inhibitors involving in cell cycle arrest. Cyclin D1 and cyclin B1

are members of cyclin protein family, which regulate cell cycle progression.[7] G2-M transition is being controlled by cyclin B1. In addition, P53 prevent the G2 to M phase transition through declining the level of cyclin B.[8]

Chemotherapeutic drugs are mostly used in the treatment of the metastatic forms of cancers, in which many of them (more than 80%) are derived from plant compounds.[9] Recently the use of natural medicines for cancer therapy has been considered. Hesa-A is a natural biological drug with marine-herbal origin, which is patented in IRAN.[10] Islamic Republic of Iran's Ministry of Health and Medical Education permitted the mass production of Hesa-A (registration number: D-5-6638; dated on 14/06/2005).[11] As it is said by the inventor of this drug, Hesa-A contains Penaeus latisculatus (king prawn), Carum carvi, and Apium graveolens. A mixture of calcium carbonate, magnesium (sulfate and phosphate), potassium (sulfate and phosphate), are sodium (sulfate and phosphate), are the mineral content of this drug. Other elements in salt or complex forms in the Hesa-A compound including: arsenic (As), bromine (Br), barium (Ba), cesium (Cs), copper (Cu), erbium (Er), lutetium (Lu), manganese (Mn), nickel (Ni), strontium (Sr), silver (Ag), thallium (Ti), tellurium (Te), thulium (Tm), titanium (Ti), tungsten (W), vanadium (Va), and zinc (Zn) .[12]

This drug has antioxidant and anticancer properties. Also it has the ability to inhibit cell growth in cancer cells.[13,14] Yet, its mechanism in esophageal cancer is not defined very well.

In this study, we used KYSE-30 cell line as human epithelial esophageal cancer cell line derived from esophageal squamous cell carcinoma. 3-(4,5-dimethlthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) assay was used to investigate the cytotoxicity of Hesa-A on esophageal malignancy cell line (KYSE-30). Flow cytometry was used to assess the effect of Hesa-A on KYSE-30 cell cycle arrest and real-time polymerase chain reaction (PCR) was used to evaluate the P53, P21, P16, cyclin D1, and cyclin B1 genes expression after Hesa-A treatment.

MATERIALS AND METHODS

Cell culture

The human esophageal carcinoma cell line, KYSE-30, was cultivated as previously described.[15] Then, 96-well flat-bottomed culture plates (TPP, Switzerland) were used to seed the cells into them for further MTT assay or 6-well plate (TPP, Switzerland) was applied for real time PCR and flow cytometry. Then they were incubated at 37°C in a 5% CO_2 incubator.

MTT assay

For MTT assay we used 5000 viable cells per well in 96-well bottom flatted plate (SPL, Korea) and incubated at 37 ºC and 5% CO_2 in a humidified environment. The cells were treated with Hesa-A in different concentrations after 24 hours, and were incubated more for additional 24 hours for MTT assay. MTT solution (0.5 mg/mL) was added to each well after the time passed and they were incubated at 37°C for 4 hours. Then, after removing the medium the blue formazan crystals were dissolved in 200µL of DMSO(Dimethyl sulfoxide) and 25µL Sorenson buffer. A microplate reader (Biotek, model Elx808) was used to read the absorbance at 570 nm. Each experiment was repeated for three times.

Flow cytometry

To assess the cell cycle arrest 300,000 viable KYSE-30 cells were cultured in 6-well tissue culture plate (SPL, Korea). After 24 hours they were treated with 0.8 mg/mL Hesa-A for 24 and 48 hours. Then, the cells were suspended in cold 70% ethanol and incubated on ice for 30 minutes. After an appropriate time, the cells were washed in PBS and RNAse A (INtRON) was added at 37°C for 30 minutes. Then, it was stained with PI (propidium iodide) in the dark. Cell population undergoing cell cycle phases was obtained by BD FACS Calibur flow cytometry and the data were analyzed using FlowJo software.

RNA extraction and c-DNA synthesis

Similar to flow cytometry, we used 300,000 viable cells per each 6-well tissue culture plate and incubated overnight. Then Hesa-A was treated for 24 and 48 hours and left in incubator. After that, the medium was removed and Cinagene Kit (RNX-Plus Solution, SinaClon, Iran) was used to extract the total RNA from each sample. CDNA synthesis kit (Qiagene) was used for cDNA synthesis.

Real-time PCR

Table 1: **Primer sequences used in real-time PCR**

Genes		Primer sequences
P53	Forward	GTT CCG AGA GCT GAA TGA GG
	Reverse	ACT TCA GGT GGC TGG AGT GA
P16	Forward	CCT CGT GCT GAT GCT ACT GA
	Reverse	CAT CAT CAT GAC CTG GTC TTC T
P21	Forward	GCT TCA TGC CAG CTA CTT CC
	Reverse	CCC TTC AAA GTG CCA TCT GT
Cyclin B1	Forward	GCC TCT ACC TTT GCA CTT CC
	Reverse	TGC TGC AAT TTG AGA AGG AG
Cyclin D1	Forward	GCG GAG GAG AAC AAA CAG AT
	Reverse	TGA ACT TCA CAT CTG TGG CA

Real-time PCR with Rotor-Gene 6000 real-time PCR Detection System (Corbett, UK) was applied to determine the effect of Hesa-A on the P53, P16, P21, cyclin D1, and cyclin B1 genes expression in esophageal carcinoma. First-strand cDNA was amplified for various mRNA, using P53, P16, P21, cyclin D1, and cyclin B1. Table 1 shows the primers used in this study. β-Actin was used for housekeeping genes.

Statistical analysis

Microsoft Office Excel 2007 software was used for statistical analysis. Results were expressed as mean, standard deviation, and correlation coefficients (R2) and were compared with the cells without treatment (control group). P values ≤ 0.05 were considered as statistically significant.

RESULTS

MTT assay

24 hours after treatment of the cells, MTT assay was performed to measure the cytotoxic effect of Hesa-A on the KYSE-30 cell lines proliferation. The human esophageal cancer cell line (KYSE-30) was treated with a variety of Hesa-A concentration (from 0.025 mg/mL to 0.8 mg/mL) and was compared with the control group (untreated KYSE-30 cell lines, figure 1).

Flow cytometry

The percentage of treated cells and controls in each phase of the cell cycle was compared using flow cytometry. As shown in figures 2A and B, Hesa-A increased the cell percentage in G2 phase after 24 and 48 hours (figures 2A and 2B).

Analysis of the paclitaxel effect on expression of P53, P16, P21

To determine the gene expression, real-time PCR analysis was done. The relative expression for each group was measured after normalization of the Ct values of P53, P21, P16, cyclin B1, and cyclin D1 genes in KYSE-30 treated cells according to the mRNA level of β-actin (as housekeeping gene). After 24 and 48 hours of Hesa-A treatment, the levels of P53, P21, P16, cyclin B1, and cyclin D1 genes increased. Level of P53 increased more than 13 times than the control group, and also its downstream gene (P21) increased significantly (more than 20 times compared with the control group, figure 3A and B).

DISCUSSION

Our study focused on the effect of Hesa-A on the esophageal carcinoma cell lines. Also cell cycle genes were investigated in this study. An arrest in G2 phase of esophageal cell cycle, was induced by Hesa-A. Also, after 24 hours P21 gene expression was raised. This raise was significant after 48 hours of treatment. P53 gene expression increased after 24 hours, too. But cyclin D1, and cyclin B1 genes expression decreased after time of treatment passed. So, in this study the potential mechanisms for G2 arrest of KYSE-30 esophageal cancer cell line by Hesa-A was through the P21 and P16 expression. After 48 hours, 2.23 times more expression of cyclin D1 gene was detected, but after 24 and 48 hours the gene expression level of cyclin B1 was less than untreated group.

The cell cycle-associated proteins are represented by P53, P21, and P16. They also play essential roles in cell proliferation and tumor cells.[16] As a suppressing tumor protein, P53 regulates and modulates some cell pathway, DNA repair, apoptosis, and cell cycle.[17] P53 affects P21 expression, and consequently P21 induction could cause cell cycle arrest and prevent tumor progression.[18] P16 could prevent cell cycle development and encourages apoptosis or growth arrest.[19] Furthermore, it has been shown that cyclin D1 down-regulation represents DNA repair, but cyclin D1 over- expression prevents DNA repair.[20]

It seems that increasing P35 expression after Hesa-A treatment, decreases the level of cyclin B. Previous studies showed that P53 prevents G2 to M phase transi-

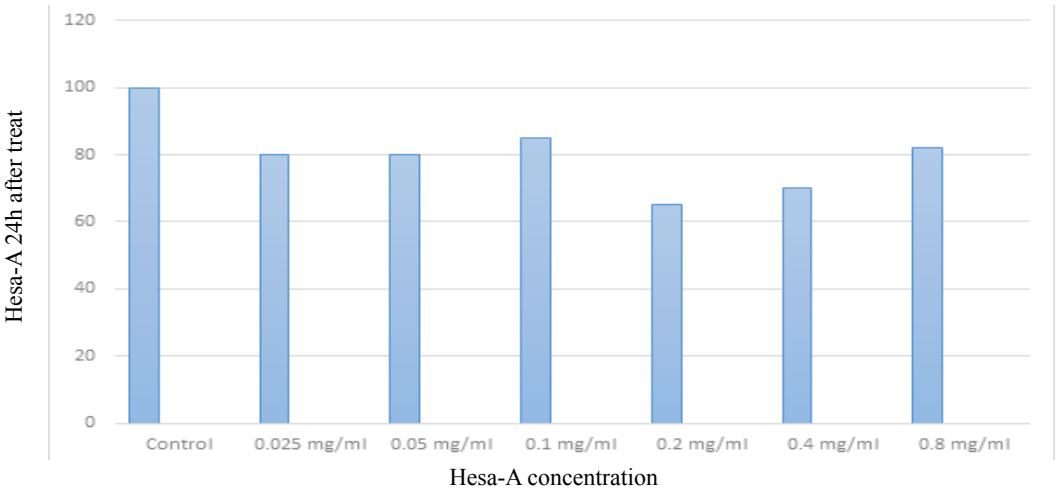

Fig.1: MTT assay measured the effect of Hesa-A on esophageal carcinoma cell line growth 24 hours after treatment. All data were normalized with control group (not treated with Hesa-A). The experiments were repeated three times.

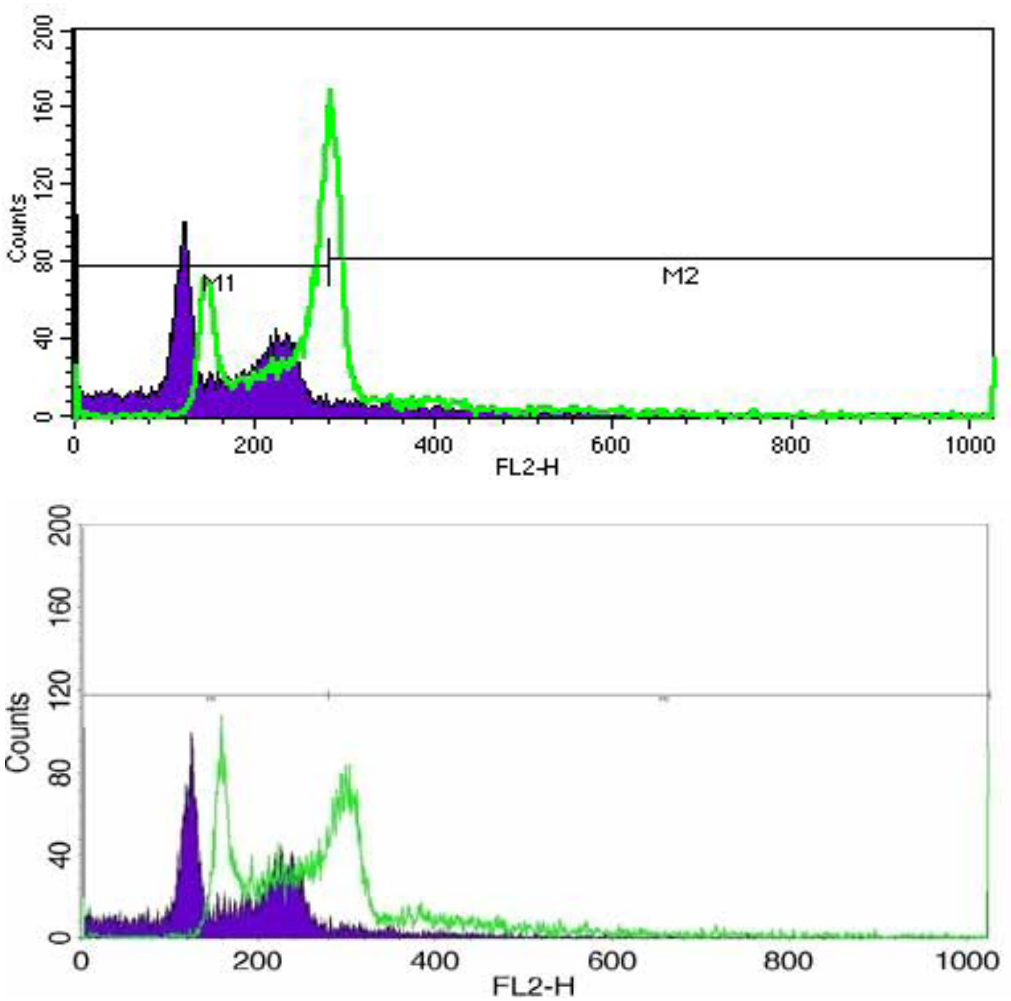

Fig.2: Hesa-A induces cell cycle arrest. Cell cycle arrest after (A) 24h and (B) 48 hours treatment with Hesa-A (0.8mg/mL) compared with the controls. Purple color shows the control cells without Hesa-A and green color represents the cell cycle arrest of Hesa-A treated cells.

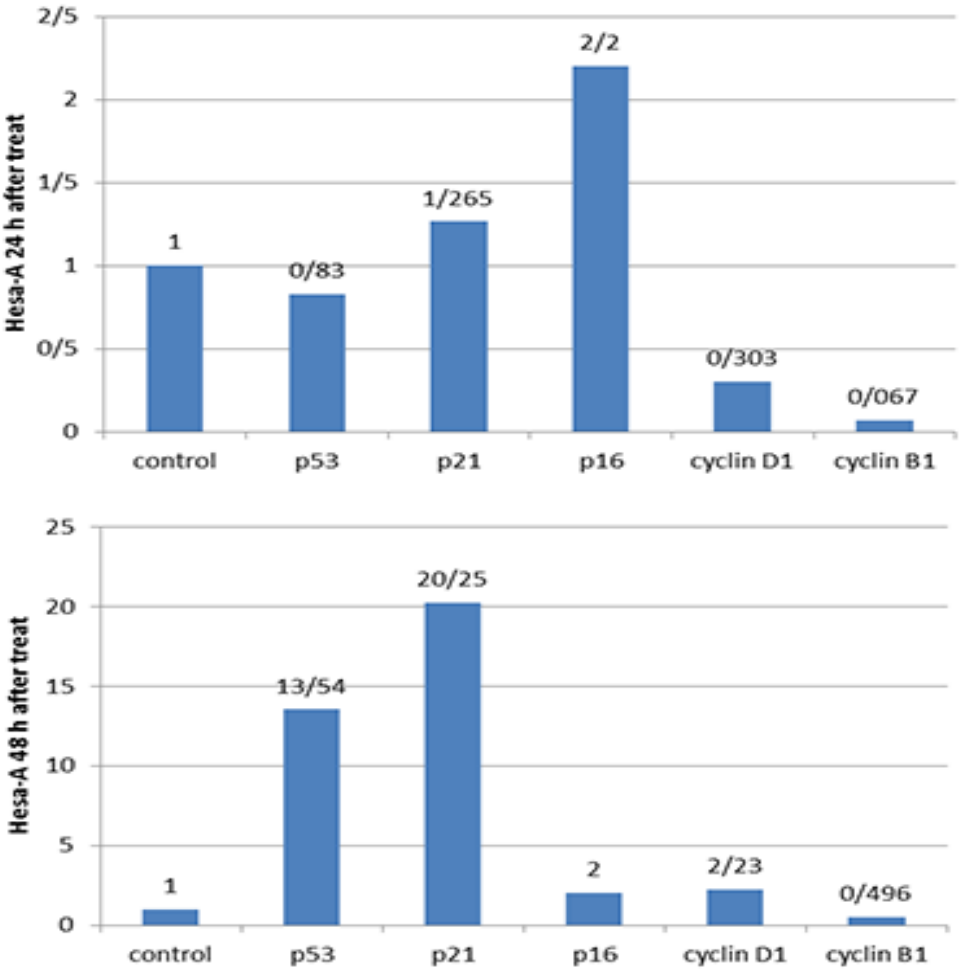

Fig.3: Real-time PCR analysis: All measured values were normalized to β-actin gene expression after (A) 24 and (B) 48 hours. The results related to increase in P53, P21, P16, cyclin B1, and cyclin D1 genes expression after Hesa-A treatment with 0.8mg/mL concentration.

tion through declining the level of cyclin B, therefore arrest of cell cycle in G2 phase may occur.[8,21,22]

Almost all previous studies in this field evaluated the effect of Hesa-A on prevention of neoplasm or decreasing cell growth and increasing apoptosis in human or animal subjects .[11] Potent cytotoxic effects of Hesa-A on different human cancer cells have been reported in a newly published paper regarding the anti-proliferative effect of Hesa-A on MCF-7 (breast adenocarcinoma), SKOV3 (ovarian cancer), PC-3 (prostate adenocarcinoma), and A549 (lung non-small cancer) cell lines.[23]

It could be suggested that P21 and P16 expressions are the main route for the cytotoxic effects of Hesa-A on esophageal cancer cells that arrest the G2 phase in the cell cycle.

Authors' contributions

Nasser Ahmadian, Roghiyeh Pashaei-Asl, Nasser Samadi, Mohammad Rahmati-yamchi, Mohammad-Reza Rashidi, Masomeh Ahmadian, Moosa Esmaeili, Faezeh Salamat, Sima Besharat and HamidReza Joshaghani have made substantial contributions to conception and design, acquisition of data, analysis and interpretation of data and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All of them also have been involved in drafting the manuscript or revising it critically

for important intellectual content and have given final approval of the version to be published.

CONFLICT OF INTEREST

The authors declare no conflict of interest related to this work.

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