THE EFFECTS OF FARNESYLTRANSFERASE INHIBITOR L-744,832 AND RADIOTHERAPY ON H-RAS+(SVR A221A) CELL LINE

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ABSTRACT

Objective: *We planned to assess the effects of two doses farnesyltransferase enzyme inhibitor L-744,832, either alone or in combination with low, medium, and high dose radiation on cultured H-Ras positive cells (SVR A221a) regarding the cell count, DNA indices and apoptosis determined by flow-cytometric method.*

Material and Method: *H-Ras positive (H-Ras +) cells were incubated. At the 3rd hour of the incubation, L-744,832 is added to study groups in following amounts. On the 3rd day of incubation, 2, 6 and 10 gray doses radiation was applied. After incubation period, study and control groups were evaluated for cell numbers, cell cycle and apoptosis percentage.*

Results: *Total count of H-Ras positive cells were significantly lower in all study groups compared with the control group, this finding was more prominent in RT2+L2, RT3, RT3+L1, and RT3+L2 groups. Apoptosis ratios were found to be similar to the control group in L1, L2, and RT1 groups, while it was significantly and similarly higher in other groups. Expect L1, the ratio of the cells in G_2/M phase was significantly higher compared to the control group. The most prominent increase was observed in RT3, RT3+L1, and RT3+L2 groups.*

Conclusion: *Possible curative effects of this agent on ras oncogene related neoplastic diseases may lead to new treatment protocols including L-744,832 alone or in combination with radiotherapy.*

Key Words: *Farnesyltransferase, H-Ras, cell line, apoptosis*
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H-RAS+ (SVR A221A) HÜCRE SERİSİ ÜZERİNE FARNESİLTRANSFERAZ İNHİBİTÖRÜ L-744,832 VE RADYOTERAPİNİN ETKİLERİ

ÖZET

Amaç: Farnesiltransferaz enzim inhibitörü L-744,832'nin iki dozunun tek başına veya düşük, orta ve yüksek doz radyasyon ile birlikte kültüre edilmiş H-Ras pozitif hücreler (SVR A221a) üzerine etkilerini değerlendirilmiştir. Hücre sayısı, DNA indeksi ve apoptoz flow sitometri ile bakılmıştır.

Materyal ve Metod: H-Ras pozitif hücreler inkübe edildi. İnkübasyonun üçüncü saatinde çalışma gruplarına L-744,832 takip eden miktarlarda eklendi. İnkübasyonun üçüncü günü 2, 6 ve 10 gray radyasyon uygulandı. İnkübasyon periyodundan sonra çalışma ve kontrol grupları hücre sayısı, hücre siklusu ve apoptoz yüzdesi için değerlendirildi.

Bulgular: H-Ras pozitif hücrelerin toplam sayısı kontrol grubuna göre tüm çalışma gruplarında anlamlı olarak düşüktü. Bu bulgu RT2+L2, RT3, RT3+L1 ve RT3+L2 gruplarında daha belirgindi. Apoptoz oranları L1, L2 ve RT1 grubunda kontrol grubu ile benzerdi.

Diğer gruplarda ise benzer şekilde yüksekti ve istatistiksel olarak anlamlıydı. L1 grubu haricinde G_2/M fazındaki hücrelerin oranı kontrol grubuna göre anlamlı olarak daha yüksekti. En belirgin artış RT3, RT3+L1 ve RT3+L2 gruplarında gözlendi.

Sonuç: Ras onkogeni ilişkili neoplastik hastalıklar üzerine bu ajanların olası küratif etkileri L-744,832'nin tek başına veya radyoterapi ile kombine olduğu yeni tedavi protokollerinin geliştirilmesine yol açabilir.

Anahtar Kelimeler: Farnesiltransferaz, H-Ras, hücre serisi, apoptoz **Nobel Med 2012; 8(3): 76-80**

INTRODUCTION

Identification of changes that play a role in the carcinogenesis process has led to find new therapeutic approaches. Oncogenes are important factors taking part in this process. Via activation of oncogenes, cancerous cells may undergo a state of uncontrolled proliferation, or differentiation sunsequently apoptosis mechanisms of these cells may be impaired.[1]

Ras is one of these oncogenes, which takes part in a wide signalling network responsible for the cell differentiation and proliferation. Ras oncogene mutation is present in 25% of human tumors and its incidence is 95% in pancreatic cancer, 67% in thyroid cancer and 47% in colon cancer.[2,3] Mutant ras gene encodes for the proteins taking part in the carcinogenesis process. Three proteins belonging to ras family play a role in the cancer: H-Ras, K-Ras and N-Ras. In order for functioning, ras protein requires being located in the cellular membrane. Blocking farnesyltransferase (FT) enzyme prevents ras protein from anchoring interior side of the membrane and becoming functional.

Since H-Ras is more dependent on FT enzyme for functioning compared to other ras proteins, studies investigating effect of FT enzyme inhibitors on H-Ras+ cells have become a current issue.

In our study, we planned to investigate effects of variable doses of L-744,382, a peptidomimetic FT enzyme inhibitor, on H-Ras+ cell lines (SVR A221a) in combination with increasing doses of radiotherapy. Possible evidence in favor of the ameliorative effects of the drug may encourage clinical studies on patients with H-Ras+ tumor.

MATERIAL and METHOD

Karadeniz Technical University Medical Faculty, Internal Medicine Department, Hematology-Oncology Laboratory conducted this study.

The study was approved by the institutional ethics committee.

Materials

Chemical materials used in this study are Dulbecco's modified Eagle's medium (Sigma, St. Louis. MO, USA), L-744,832 (Sigma), fetal calf serum (Sigma), 100U/mL penicillin (Llorente Laboratories, Madrid, Spain), 100 µg/mL streptomycin (Llorente Laboratories), trypsin (Sigma), Coulter DNA prep reagents (DNA prep stein, DNA prep LPR)(Coulter, San Diago, California, USA), physiologic serum (SF) 0.9%, PBS (Oxoid Ltd, Basingstoke, Hampshire, England).

Derivation of H-Ras+ Cells and Preparation for The Culture

H-Ras+ cell line (SVR A221a) used in this study was bought from ATCC (American Type Culture Collection). SVR A221a cells were tumorigenic cell lines derived from pancreatic islet endothelium tissue of *Mus musculus* (mouse).[4] Dulbecco's modified →

Table 1: Study Groups		
Groups	**Explanations**	**Abbreviations**
Group 1	Control Group	
Group 2	L-744,832 2μM/L	L1
Group 3	L-744,832 20μM/L	L2
Group 4	Radiation 2 gray	RT1
Group 5	Radiation 2 gray + L-744,832 2μM/L	RT1+L1
Group 6	Radiation 2 gray + L-744,832 20μM/L	RT1+L2
Group 7	Radiation 6 gray	RT2
Group 8	Radiation 6 gray + L-744,832 2μM/L	RT2+L1
Group 9	Radiation 6 gray + L-744,832 20μM/L	RT2+L2
Group 10	Radiation 10 gray	RT3
Group 11	Radiation 10 gray + L-744,832 2μM/L	RT3+L1
Group 12	Radiation 10 gray + L-744,832 20μM/L	RT3+L2

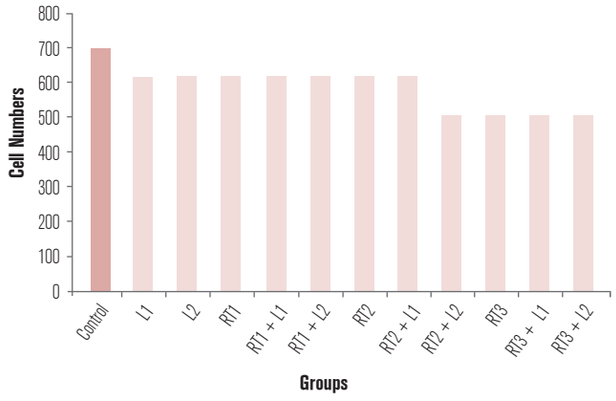

Figure 1. Cell numbers in control and study groups.

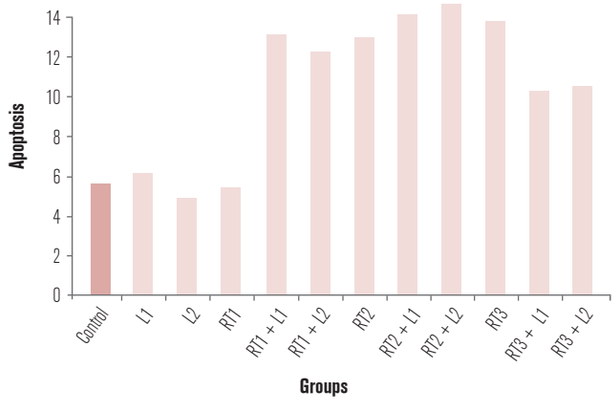

Figure 2. Apoptosis ratios (%) in control and study groups.

Eagle's medium is used as culture medium for these cells which have an adherent structure and do not contain MAPKK gene.

The cells, which were brought to the laboratory in cold chain and stored at -80°C, were defrosted pre-experimentally at room temperature. RPMI containing 10% fetal calf serum, 100 unit/ml penicillin and 100 μg/ml streptomycin was added to the cells transferred to a cubic tube and then, 10 minutes centrifugation at 800g was performed. Following centrifugation, supernatant was removed from the medium and precipitated cells were homogenized with RPMI culture medium. This procedure was repeated three times. Derived cells were allowed to proliferate in a 25 ml cell culture flask.

L-744,832

Farnesyltransferase inhibitor L-744,832 that we used in our study was bought from Sigma and stored at 4°C in a freezer. L-744,832 was defrosted pre-experimentally in a phosphate-buffered saline (PBS). Prepared stock solutions were stored at -20°C and protected from light.

H-Ras+ (SVR A221a) Cell Culture

H-Ras+ cells (SVR A221a) were incubated in a 5% CO_2 medium at 37°C until the ground of the culture flask became 75% confluent. Culture medium was added once in three days. In our study, a cell culture medium containing Dulbecco's modified Eagle's medium (4 mM L-glutamine, 1.5 g/L sodium bicarbonate and 1 g/L glucose), 10% inactive fetal calf serum, 100 unit/mL crystallized penicillin and 100 μg/ml streptomycin was used. When the ground of 25 ml cell culture flask had become 75% confluent, trypsinization process was applied to the cells by using 0.25% trypsin prepared with PBS. After the trypsinization process, SVR A221a cells transferred to a cubic tube were converted to a cell suspension with RPMI 1640 culture medium.

The cells in the culture medium were distributed to the wells of the culture plate ensuring that in each well, the ultimate volume was 1 ml and 500.000 cells were present. Plates were allowed to incubate in 5% CO_2 incubator. At the 3rd hour of the incubation, L-744,832 is added to study groups in following amounts.[5] On the 3rd day of incubation, 2, 6 and 10 gray doses radiation was applied to the corresponding groups in Radiation Oncology Department of our hospital. Study groups are summarized in Table 1.

Culture media of the cells cultivated in plates were renewed on the 3rd day of the incubation and the drug was re-added in appropriate doses. On the 7th day of the incubation, trypsinization process was applied to incubated cell culture groups. After the trypsinization, the derived cells were evaluated as follows:

Evaluation Method

After incubation, derived cells were evaluated in terms of following parameters. →

Cell Number

Following 6 days incubation, trypsinization process was applied. Number of cells in each derived group was counted by using blood counter.

Detection of DNA Index and Apoptosis with Flow Cytometry

Percentage of apoptosis and percentage of cells in G_0/G_1, G_1/M and S phase was measured. For flow cytometric analysis, cells derived after incubation were washed with PBS and cell numbers were adjusted as being $5x10^7$/mL. G_0/G_1, G_2/M, S phases of the cell cycle were evaluated as percentage by using multi-cycle DNA analysis program.[6]

Immunotech (A Beckman Coulter Company) Annexin 5-FITC kit (PN IM 3546) (containing Annexin 5-FITC solution, concentrated binding buffer and propidium iodide) was used to find out percentage apoptosis in all groups. Following incubation and necessary preparatory steps, study tubes were evaluated with flow cytometry. Apoptosis, which appeared as hypodiploidic peak (DNA index <1) in flow cytometry, was evaluated as percentage.

RESULTS

Cell Numbers

H-Ras+ cells (SVR A221a) were evaluated on 6th day of incubation. Before evaluation, photographs of cells in various wells were taken with inverted microscope. It was observed that cell numbers had decreased in all study groups in comparison with the control group. Decrease in cell numbers was more evident in RT2+L2, RT3, RT3+L1, RT3+L2 groups than other study groups (Figure 1).

Apoptosis Ratios

Apoptosis ratios of L1, L2, RT1 groups were similar to that observed in the control group. Apoptosis ratios observed in RT1+L1, RT1+L2, RT2, RT2+L1, RT2+L2, RT3, RT3+L1, RT3+L2 groups significantly increased compared to the control group. Among these groups, apoptosis ratios were similar to each other (Figure 2).

Cell Cycle Analysis

Except for L1 group, ratio of cells in G_2/M phase increased in all study groups compared to the control group. The most significant increase was observed in RT3, RT3+L1, RT3+L2 groups. Except for L1 group, ratio of cells in G_0/G_1 phase decreased in all

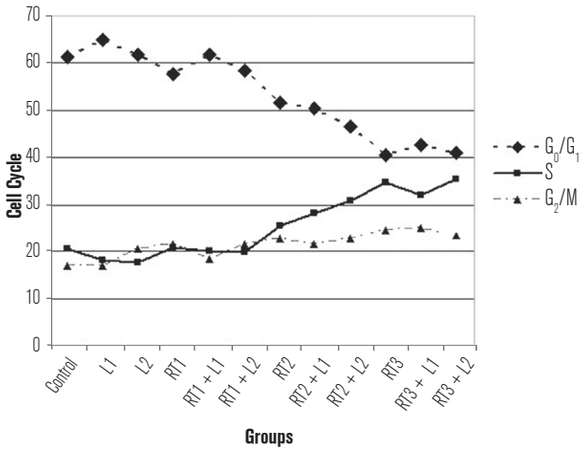

Figure 3. Cell cycle analysis (%) in control and study groups.

study groups compared to the control group. The most significant decrease was observed in RT2+L2, RT3, RT3+L1, RT3+L2 groups. The ratio of cells in S phase decreased in L1, L2, RT1+L1, RT1+L2 groups compared to the control group while it is similar to the control group in RT1 group. In all other groups (especially in RT3, RT3+L1 and RT3+L2), ratio of cells in S phase increased compared to the control group (Figure 3).

DISCUSSION

This study is the first trial to investigate effects of FT inhibitor on H-Ras transformed SVR A221a mouse endothelial cells. In this study, we observed that L-744,832 affected the number of H-Ras+ cells in a dose-dependent manner. The evaluations made on the 6th day revealed that number of cells in the examined cell line decreased as the dose of the drug was increased.

It is not clear if inhibition of farnesylation can break the radiation resistance. Evidences from first studies showed that it was dependent on the cell line and FT inhibitor used. Generally, favorable results were obtained in cell lines with H-Ras mutation.[7] On the other hand, resistance could not be broken in cell lines with K- and N-Ras mutations.[7-9] It has been reported that H-Ras blockade could decrease O_2 consumption via reducing the rate of cell proliferation and this effect has led to reduced tumor hypoxy. As a result, radiosensitive effect could be arisen.[10] In tumors with mutations other than H-Ras, radiosensitivity could be obtained only when FT inhibitors are applied in combination with GGT-I inhibitors.[8]

Combined therapy of radiation and L-744,832 was experimented only in one animal study and it was reported that synergic effect was observed when radiation was administered in combination with →

L-744,832 to animals implanted with human tumors which were mutant for H-Ras.

PI 3K and Raf, two Ras effectors, were reported to be responsible for radiation resistance.[10-12] Blockade of H-ras farnesylation adequately inactivates these pathways. There is no sufficient evidence to propose that the proteins (such as CNEP-E and CNEP-F) that are subject to mandatory farnesylation may play a role in radiation resistance. On the other hand, it is considered that they may play an active role in sensitivity to FT inhibitor.[13] No previous cell line study investigating combined effect of radiation and L-744,832 is present in the literature. In our study, which is the first one in its scope, three different radiation doses (2, 6 and 10 gray) were used. Administration of low dose radiation alone or in combination with L-744,832 did not have a major effect on cell proliferation. Current cytometry revealed that 62.5% of cells were in G_0/G_1 phase, indicating a reduction in rate of proliferation. It is known that cells with low rate of proliferation show a low sensitivity to radiation.[14] Therefore, it is reasonable to propose that low dose radiation was not effective on number of cells.

We observed that administration of 2-gray radiation alone did not result in any increase in apoptosis, but addition of L-744,832 increased the apoptosis independent from the dose. This increase resulted in a decrease in number of cells, because intensity of the effect may not be strong enough. In 6- and 10-gray radiation groups, addition of L-744,832 did not have any effect on the increase in apoptosis and the decrease in number of cells. Synergic effect on apoptosis was observed only in low dose radiation group.

It was previously reported that radiation results in G_2/M accumulation and despite the presence of radiation, cells complete the cycle with a delay.[15] Besides, combination of radiation and FT inhibitor may exert its effects through G_2/M accumulation via blockade of farnesylation of lamin B, CENP-E and CENP-F.[7] In our study, a mild increase in G_2/M blockade was observed as the radiation dose increased. Addition of L-744,832 did not increase G_2/M blockade.

CONCLUSION

Ras oncogenes play an important role in the carcinogenesis process that constitutes 25% of human tumors.[2,3] Although successful results were obtained with the administration of FT inhibitors on tumors with H-Ras mutations in vitro conditions, its effect on proteins other than H-Ras is more restricted. Results of our study should be reinforced with more comprehensive in vitro studies and animal studies. Possible favorable results can constitute a background for the design of new clinical studies investigating effects of L-744,832 alone or in combination with radiotherapy on the cancer patients that ras oncogene actively takes part in the pathogenesis of the disease.

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