ORIGINAL ARTICLE

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Effect of Pro-Inflammatory Cytokine IL-1β, on Urotensin II Gene Expression in Human Lung Cancer Cells

ABSTRACT

Objective: Lung cancer is the deadliest cancer type world-wide. Poor prognosis of lung cancer patients and lack of an effective treatment require detailed understanding of lung cancer pathogenesis. It was highlighted in some studies that U-II is likely to be a biomarker or molecular target for the prevention and treatment of some diseases such as lung cancer. But its molecular action mechanism has not been elucidated yet. In the present study, we aimed to investigate the role of U-II in lung cancer.

Methods: In our study, A549 cells were induced with different doses of IL-1β at different durations (1, 3 ng/ml; 6, 24 hours). mRNA levels of GAPDH, NF-κB1, MMP-1, and U-II were analyzed with RT-qPCR. The Delta Delta Ct (ΔΔCt) method was used for data analysis. The analyzed data were expressed as the "fold-change".

Results: Our results indicate that U-II gene is expressed in A549 cells and IL-1β can induce gene expressions of U-II, MMP-1 and NF-κB1 in A549 cells.

Conclusions: U-II is a promising molecular target in treatment and prevention of lung cancer. Therefore, further studies are needed to enlighten molecular mechanism of U-II in lung adenocarcinoma.

Keywords: Urotensin II, IL-1β, NF-κB1, MMP-1, Lung Cancer

Pro-Enflamatuvar Sitokin IL-1β'nın İnsan Akciğer Kanseri Hücrelerinde Urotensin II Gen Ekspresyonu Üzerine Etkisi

ÖZET

Amaç: Akciğer kanseri dünya çapında en ölümcül kanser türüdür. Akciğer kanseri hastalarının kötü prognozu ve etkili tedavinin olmaması akciğer kanseri patogenezinin ayrıntılı olarak anlaşılmasını gerektirir. Bazı çalışmalarda, U-II'nin akciğer kanseri gibi bazı hastalıkların önlenmesi ve tedavisinde olası biyobelirteç veya moleküler hedef olma durumu vurgulanmaktadır. Ancak, U-II'nin moleküler aksiyon mekanizması henüz aydınlatılmamıştır. Bu çalışmada, akciğer kanserinde U-II'nin rolünü araştırmayı amaçladık.

Gereç ve Yöntem: Çalışmamızda A549 hücre dizileri farklı doz ve sürelerde IL-1β ile uyarıldı (1, 3 ng/ml; 6, 24 saat). GAPDH, NF-κB1, MMP-1 ve U-II mRNA seviyeleri RT-qPCR yöntemi ile analiz edildi. Verilerin analizinde delta delta CT (ΔΔCt) metodu kullanıldı. Analiz edilen veriler 'katlı değişim' olarak ifade edildi.

Bulgular: Bulgularımız, A549 hücrelerinde U-II geninin eksprese edildiğini ve A549 hücrelerinde IL-1β'nın U-II, MMP-1 ve NF-κB1 gen ekspresyonlarını indükleyebildiğini göstermektedir.

Sonuç: U-II, akciğer kanseri tedavisinde ve önlenmesinde umut verici bir moleküler hedeftir. Bu nedenle, akciğer adenokarsinomasında U-II'nin moleküler mekanizmasını aydınlatmak için daha ileri çalışmalara ihtiyaç vardır

Anahtar Kelimeler: Ürotensin II, IL-1β, NF-κB1, MMP-1, Akciğer Kanseri

INTRODUCTION

Lung cancer is the most prevalent tumor type in the world and the primary cause of cancer-related deaths world-wide (1, 2). Each year, almost 1.8 million people are diagnosed with lung cancer and 1.6 million of them die due to this highly fatal disease (1). In the studies including up to date, the relation between cancer and inflammation has been intensely investigated and it is reported that inflammation is an important compound of the cancer (3-7). Hussain and Harris reported that chronic inflammation and infections contribute to about 25% of all known human cancer types (6). Lung cancer is a typical cancer that is associated with inflammation (8, 9). For instance, smoking can cause chronic inflammation and carcinoma in lungs (7). Inflammatory mediators residing tumor micro-environment play a central role in tumor progression (10). IL-1β (Interleukin 1 beta) is an important pro-inflammatory cytokine implicated in many diseases including lung cancer (11-13). Besides, IL-1β is considered as tumorigenesis-inducing cytokine. Studies indicated that IL-1β has a role in augmented growth, metastasis and angiogenesis of tumors (14-16). It can be stated that inflammatory stimulants have an evident contribution to the progression of the lung cancer. But the underlying pathogenic molecular mechanism has not been elucidated yet.

Urotensin II (U-II), a somatostatin-like cyclic peptide, was first isolated from the urophysis of the fish and is defined as one of the strongest vasoconstrictor agents today (17, 18). U-II is the endogenic ligand of the orphan G protein-coupled receptor (GRP14) which is also known as U-II receptor (UT-R) (17, 19). In previous studies it was reported that U-II involves in the pathophysiology of some tumor types. In a study conducted by Takahashi et al., the presence of U-II and UT-R mRNA expressions were detected in several human cancer cell lines (20). Furthermore, in other studies, in vivo mRNA expressions of U-II and UT-R genes were observed in adrenal tumor and renal carcinoma patients (21, 22). The expression pattern of U-II and UT-R genes in human lung adenocarcinoma cell line was also demonstrated previously. Additionally in the same study, it was shown that U-II could stimulate cell proliferation in A549 cell line and also induce tumor growth in vivo in a nude mouse xenograft model (23). Furthermore, in a study with another nude mouse tumor model, it was stated that U-II and UT-R antagonists can have a potential role in treatment and prevention of lung cancer (24). Along with these findings, we still need further studies for a better understanding of the role of U-II in lung cancer. For this purpose, we aimed in our study to observe how the expression level of U-II is regulated in IL-1β-induced A549 cancer cell line.

MATERIAL AND METHODS

Cell Culture and Reagents: We obtained human lung adenocarcinoma cell line A549 from the American Tissue Type Collection (ATTC, Manassas, VA). A549 cells were cultured in DMEM with 10% FBS and 1% penicillin/streptomycin in a humidified 37˚C incubator with 95% air and 5% CO2. During experimental stage, the cells were transferred into serum-free culture medium after they reached %70-80 confluence. Then A549 cells were stimulated with IL-1β (R&D, 201-LB) at different doses (1, 3 ng/ml) and time intervals (6h, 24h).

RNA Isolation and Quantitative Real-Time RT-PCR: Total RNA was isolated from cell cultures using miRNeasy mini kit (Qiagen, Hilden, Germany). Then, RNA concentration was determined spectrophotometrically (MultiskanTM GO, ThermoFisher, FINLAND). cDNA was synthetized with one-step RT-PCR by using RT2 HT First Strand Kit (Qiagen, Maryland, USA). Primers specific for interested genes (U-II [Catalog No: PPH02516A, NCBI RefSeq: NM_006786.3], NF-κB1 (Nuclear factor kappa B1) [Catalog No: PPH00204F, NCBI RefSeq: NM_001165412.1], MMP1 (Matrix metallopeptidase 1) [Catalog No: PPH00120B, NCBI RefSeq: NM_001145938.1], GAPDH (Glyceraldehyde-3-phosphate dehydrogenase, housekeeping gene) [Catalog No: PPH00150F, NCBI RefSeq: NM_001256799]) were purchased from Qiagen. The reaction mixture for each gene qPCR contained 1 µl cDNA template, 1 µl of the each primers (UII, NF-κB1, MMP1 and GAPDH), 12,5 µl RT2 SYBR Green Master Mix (Qiagen, Maryland, USA) and 10,5 µl RNase-free water in a total reaction volume of 25 µl with 95 °C for 10 min, followed by 40 cycles of 95°C for denaturation for 15 sec, and annealing/extension at 60°C for 1 min in Rotor Gene Q Real-Time PCR System (Qiagen, Hilden, Germany).

Statistical Analysis: All gene expression studies were performed in triplicate. The Gene Globe Data Analysis Center (Qiagen, online service) was used to analyze real-time PCR data. The Delta Delta Ct (ΔΔCt) method was used for data analysis by normalizing the raw data with housekeeping gene GAPDH as control. The analyzed data were expressed as the "fold-change" (2^(-ΔΔCt)) which was calculated by dividing the normalized gene expression (2^(-ΔCt)) in the unknown samples with the normalized gene expression (2^(-ΔCt)) in the control group. A criterion of fold change > 2 was considered as upregulated and <1 is as downregulated. The p values were calculated based on a student's t-test by using replicate 2^(-Delta Ct) values for each gene in the control group and treatment groups. p values less than 0.05 were considered as significant. Data were presented as mean±SD. GraphPad Prism

version 6.0 (GraphPad Software) was used to prepare graphics and Adobe Photoshop C5.1 software to design and prepare art work.

RESULTS

Effect of IL-1β on gene expression level of U-II in A549 cells: In order to observe the effect of pro-inflammatory cytokine IL-1β on U-II in A549 lung adenocarcinoma cell line, we analyzed expression levels of U-II with RT-qPCR method. As depicted in Fig 1. a, U-II expression levels vary depending on dosage and treatment duration of IL-1β. Especially at 24h after 1 and 3 ng/ml IL-1β treatments, we observed a significant increase in U-II expression levels (Fig 1. a). There was also an increase in U-II expression level at 6h after 1 ng/ml IL-1β treatment but it was lower than the one at 24h and not significant (Fig 1. a).

Effect of IL-1β on expression level of MMP-1 ve NF-κB1 in A549 cells: We also analyzed the expression levels of MMP-1 and NF-κB1 in IL-1β-induced A549 cell line with RT-qPCR. We similarly observed that gene expression levels of MMP-1 and NF-κB1 altered depending on dosage and treatment periods of IL-1β. We found that, MMP-1 mRNA levels increased significantly at 6h and 24h after treatment with 1 and 3 ng/ml IL-1β (Fig 1. b). Besides, at time periods of 6h and 24h, MMP-1 levels after 3 ng/ml IL-1β treatment were greater than the ones after 1 ng/ml IL-1β treatment (Fig 1. b). The highest increase in MMP-1 levels was observed at 24h after 3 ng/ml IL-1β treatment (Fig 1. b).

We checked also NF-κB1 gene expression levels and we found that at 6h and 24h after 1 and 2 ng/ml IL-1β treatment, NF-κB1 mRNA levels significantly increased (Fig 1 b). 3 ng/ml doses of IL-1β was more effective than 1 ng/ml and the NF-κB1 expression level picked at 6h after treatment with 3 ng/ml IL-1β (Fig 1. c).

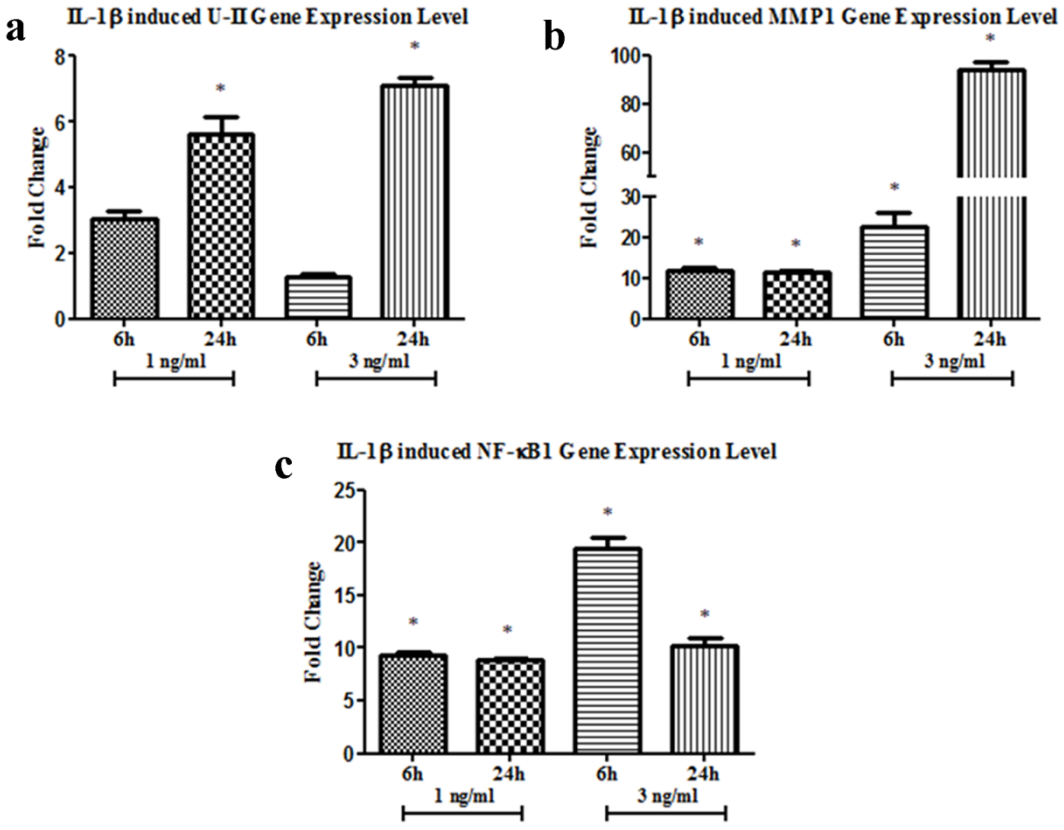

Fig 1. Gene expression levels of U-II (a), MMP-1 (b), and NF-κB1 (c) after IL-1β induction in A549 cell line. Two different concentrations of IL-1β (1 and 3 ng/ml) and two different treatment durations (6h and 24h) were depicted in different motifs on graph bars. The gene expression levels were indicated as fold change compared to the control without treatment. *$p<0.05$. Data are presented as mean±SD.

DISCUSSION

In our study, to our knowledge we showed for the first time that U-II gene expression can be induced via IL-1β treatment in A549 human lung adenocarcinoma cell line. Besides, our results point out that U-II can have a role in inflammation related tumor progression. Since it has been discovered, basic and clinical researches evaluating physiological and pathophysiological roles of U-II carry on. However, studies focusing on the function of U-II in lung cancer are at limited number and the

precise action mechanism of U-II has not been uncovered yet.

Previous studies showed that, U-II and UT-R expression were detected in some tumor tissues such as adrenal tumors, renal carcinoma, pheochromocytoma, and breast cancer (20-22, 25, 26). Moreover, in an in vitro study, the presence of U-II and UT-R expression patterns were detected in several cancer cell lines, including glioblastoma, neuroblastoma, choriocarcinoma, adrenocortical carcinoma, colorectal carcinoma, and cervical carcinoma (27). Wu et al. showed in their study for the first time the expression patterns of U-II and UT-R at both gene and protein levels in human lung adenocarcinoma cell line A549 (23). It is not only expressed in tumor cells, but U-II is also considered as a growth propagating factor in some tumor types. Previous studies demonstrated that, U-II can induce cell proliferation in adrenocortical carcinoma, renal cell carcinoma, pheochromocytoma, and lung adenocarcinoma (A549) cancer cell lines (20, 23, 26, 27). Regarding lung cancer, Wu et al. showed that different concentrations of U-II augmented the tumor cell proliferation and tumor tissue growth both in vitro with an A549 cell culture model and in vivo with a xenograft mouse model (23).

Inflammation is another dimension during tumorigenesis and tumor progression. IL-1β, a prominent pro-inflammatory cytokine involved in many diseases including lung cancer, was reported to contribute to tumor progression (11-13, 16, 28). It was stated in previous studies that U-II can also be implicated in the inflammatory processes (29, 30). In one of these studies, Johns et al. showed that U-II triggered the secretion of IL-1β in cardiomyocytes via the signaling partway through UT-R (29). In another study, Zhou et al. recognized that U-II can stimulate the secretion of inflammatory cytokines (IL-6, TNF-α, MMP-9) residing within tumoral microniches which contribute to growth, metastasis and angiogenesis of tumor cells (24). However, there have been no studies so far investigating the effect of IL-1β on U-II expression in A549 cell line.

In our study, we determined the gene expression levels of U-II, MMP-1 and NF-κB1 in IL-1β-induced A549 cells with RT-qPCR method. Our results indicated that, U-II gene expression levels increased significantly after IL-1β induction at both 6h and 24h time points in A549 cells. However, 3h induction with IL-1β did not cause any significant change in U-II expression level in A549 cells (data not shown). When we evaluate our results in the light of the findings of aforementioned studies, IL-1β can exert its effects by means of U-II in lung adenocarcinoma pathogenesis. But nevertheless, further studies will be needed to understand exact mechanism of this signaling pathway.

Another marker that we checked its gene expression level, MMP-1, functions in tumor progression and invasion. MMP-1 overexpression was implicated especially with lung cancer (31, 32). Inflammatory cytokines, like IL-1β, can contribute to tumor invasion by promoting MMP-1 expression (33, 34). In another study, Armstrong et al. demonstrated that MMP-1 expression levels altered in a time dependent manner in IL-1β-induced A549 cell line and reached its pick value at 16h after IL-1β treatment and still showed a significant increase after 24h-IL-1β-induction compared to the control group (35). Similarly in our study, the max increase in MMP-1 gene expression level was reached at 24h time point post-induction with IL-1β.

The gene expression of the last parameter we checked was NF-κB1 which is another factor involving in cellular response of inflammation. NF-κB1 is a nuclear protein that functions in progression of several human tumor types including lung cancer (36). We found almost similar expression pattern for NF-κB1 like U-II and MMP-1. The most striking difference of NF-κB1 was its pick value at 6h after 3 ng/ml IL-1β treatment whereas U-II and MMP-1 expression levels gave a pick at 24h after 3 ng/ml IL-1β treatment. As we expected, NF-κB1 acts as an initial front line inflammatory factor after induction with a pro-inflammatory cytokine IL-1β (37).

CONCLUSION

It can be concluded based on our findings that U-II gene is expressed in A549 adenocarcinoma cell line and U-II gene expression can be promoted by IL-1β stimulation. Therefore, U-II can be a promising molecular target in therapy and prevention of lung cancer. As a next step, our results should be consolidated together with different doses and treatment durations of IL-1β in order for a better understanding of the molecular mechanism of U-II in lung adenocarcinoma.

Conflicts of Interest: All authors declare that there is no conflict of interest.

Contribution of Authors: H.M.O. and M.Y.T. conceived and designed the study. H.M.O., M.Y.T., and M.U.K. wrote/drafted/edited the manuscript and interpreted the results. H.M.O., M.Y.T. and C.O.G. performed experiments and collected data. H.M.O and M.Y.T conducted analyses, prepared graphs/figures and revised the manuscript. All authors approved the content of this manuscript.

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