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ARAŞTIRMA / RESEARCH

Role of hypoxia and embryonic stem cell genes in head and neck cancers

Baş boyun kanserlerinde hipoksi ve embriyonik kök hücre genlerinin rolü

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Abstract

Purpose: The aim of this study was to determine the effect of hypoxia on HIF-1α, HIF-2α, SOX2, OCT4, NANOG, CD133, ESRRA genes in Hep-2 laryngeal and FaDu pharyngeal cancer cell lines.
Materials and Methods: Hep-2 and FaDu cell lines were exposed to hypoxia for 6, 12, 24, 48, and 72 hours to determine the effect of hypoxia and then treated with 5μM, 10μM and 20μM XCT790 under hypoxic conditions. Taqman Gene Expression Assays were used to determine mRNA expressions.
Results: In the hypoxic environment, we observed that HIF-2α, SOX2, OCT4, NANOG, CD133, and ESRRA mRNA expressions increased, except HIF-1α in Hep-2 cells. In FaDu cells, it was observed that HIF-1α and SOX2 mRNA expressions decreased, while HIF-2α, OCT4, NANOG, CD133, and ESRRA mRNA expressions increased. When cells were treated with the ESRRA inverse agonist XCT790, the hypoxia-induced gene expression profile was found altered.
Conclusion: In our study, it was determined that hypoxia changed the gene expression profile in Hep-2 and FaDu cell lines. According to our results, it was concluded that the ESRRA gene may be an important cofactor of the hypoxic response, but its inhibition alone may not be sufficient to abolish the hypoxic response. Protein data is needed to reveal the precise mechanisms of ESRRA and hypoxia association in head and neck cancers.

Keywords: Head and neck cancers, hypoxia, embryonic stem cell marker, HIF

Öz

Amaç: Bu çalışmada, Hep-2 larinks kanseri ve FaDu farenks kanseri hücre serilerinde hipoksinin, HIF-1α, HIF-2α, SOX2, OCT4, NANOG, CD133, ESRRA genleri üzerindeki etkisinin belirlenmesi amaçlanmıştır.
Gereç ve Yöntem: Hep-2 ve FaDu hücre hatları 6, 12, 24, 48 ve 72 saat süreyle hipoksiye maruz bırakılarak hipoksinin genler üzerindeki etkisi belirlendi daha sonra hücreler hipoksik koşullar altında 5μM, 10μM ve 20μM XCT790 ile muamele edildi. Genlerinin mRNA seviyeleri Taqman gen ekspresyon assayler kullanılarak belirlendi.
Bulgular: Hipoksik ortamda Hep-2 hücrelerinde HIF-1α dışında HIF-2α, SOX2, OCT4, NANOG, CD133 ve ESRRA mRNA ekspresyonlarının arttığını gözlemledik. FaDu hücrelerinde ise HIF-1α ve SOX2 mRNA ifadeleri azalırken, HIF-2α, OCT4, NANOG, CD133 ve ESRRA mRNA ifadelerinin arttığı gözlendi. Hücreler ESRRA invers agonisti XCT790 ile muamele edildiğinde, hipoksinin indüklediği gen ekspresyon profilinin değiştiği saptandı.
Sonuç: Çalışmamızda hipoksinin, Hep-2 ve FaDu hücre hatlarının gen ekspresyon profilini değiştirdiği saptanmıştır. Sonuçlarımıza göre, ESRRA geninin hipoksik yanıt oluşumunda önemli bir kofaktör olabileceği, ancak tek başına inhibisyonunun hipoksik yanıtı ortadan kaldırmak için yeterli olmayabileceği sonucuna varılmıştır. Baş ve boyun kanserlerinde ESRRA ve hipoksi ilişkisinin mekanizmalarını ortaya çıkarmak için protein verilerine ihtiyaç vardır.

Anahtar kelimeler: Baş boyun kanserleri, hipoksi, embriyonik kök hücre marker, HIF

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INTRODUCTION

Head and neck cancers describe the epithelial malignancies of the oral and nasal cavities, paranasal sinuses, pharynx, and larynx. They constitute 6% of all cancers, cause 5% of cancer deaths, and rank 6th among all cancers by frequency[1,2,3].

In recent years, the tumor microenvironment has been considered as an important factor in malignant progression and metastasis. One of the most common microenvironmental stresses and features of solid tumors is hypoxia[4]. Hypoxia is an important factor that affects tumor cell proliferation and malignant progression[5]. Adaptive response to hypoxia is provided by HIFs (Hypoxia-Inducible Factors), which are the main regulators of hypoxic gene expression and oxygen homeostasis. HIF is a heterodimeric transcription factor, belonging to the PAS family (PER, AHR, ARNT, and SIM), consisting of an oxygen-regulated alpha (HIF-1α and HIF-2α) and continuously expressed stable HIFβ subunits[6].

Studies with pluripotent cells show increased proliferation at low oxygen levels. The reduction of oxygen concentration below the physiological level, ensures the continuity of human embryonic stem cells in vitro, decreases the spontaneous differentiation and supports self-renewal[7]. Embryonic stem cells (ESC) and cancer cells (CC) have several characteristics in common, such as the ability to produce benign or malignant tumors, having a rapid cell cycle, and high Telomerase activity[8]. These common features suggest that both groups may use similar pathways in the regulation of gene expression. In embryonic stem cells, undifferentiated state is maintained by OCT4, NANOG, and SOX2 core transcription factors[9]. Studies show that ESC marker genes which are expressed in different cancer tissues and cancer cell lines[10,11,12,13], increase proliferation, inhibit apoptosis, trigger invasion, and metastasis[14,15,16]. CD133 is one of the key biomarkers isolated from stem cells, and recent studies have shown that it is involved in cell growth, development, and tumor biology[17].

Estrogen-related receptors (ERRs) have been defined as important cofactors in the HIF-mediated hypoxic response. ERRs recognize functional HIF heterodimers. Since HIFs are difficult to block by drugs, it is thought that gene expression and malignant phenotype induced by hypoxia can be eliminated by ERR inhibition[18]. Different studies have shown that hypoxia increases expression of ERRα[19] and ERRα interacts with HIF-1α[20] and HIF-2α[21] that contributes to the formation of the hypoxic response. Previous studies have shown that hypoxia induced gene expression profile can be changed by ERRα protein degradation mediated by the ERRa inverse agonist XCT790[20,21].

This study aimed to determine the effect of hypoxia on HIF-1α, HIF-2α, SOX2, OCT4, NANOG, CD133, ESRRA (ERRα) genes in Hep-2 laryngeal cancer and FaDu pharyngeal cancer cell line. Then, with the treatment of ESRRA inverse agonist XCT790, we intended to determine whether there was a change in the gene expressions induced by hypoxia.

MATERIALS AND METHODS

Cell culture

Hep-2 and FaDu cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Lonza Verviers, Belgium) supplemented with 10% Fetal Bovine Serum (FBS, Multicell, Wisent Inc.), 1% Penisilin-Streptomisin (PS, Multicell, Wisent Inc.), and 1% L-Glutamine (L-Glu Multicell, Wisent Inc.) at 37°C with 5% CO_2. Since the cells are purchased commercially, ethics committee approval is not required.

Hypoxia treatment

Hypoxic conditions were generated by the Anaerogen compact system (Anaero Gen Compaq AN0010C, Oxoid, Cambridge, UK). Cells were counted on the Thoma slide, and 100,000 cells per plate were seeded and cultured, then expected to reach 75% confluency. Plates were placed in the plastic pouch (provided by the company) together with the AnaeroGen Compact foil sachet and sealed immediately. Cells were exposed to hypoxia for 6, 12, 24, 48, and 72 hours at 37°C, 5% CO_2 incubator with the control cells (without hypoxia).

XCT790 treatment

Hep-2 and FaDu cells were counted and 100,000 cells were seeded in culture plates. The ERRa inverse agonist XCT790 (Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO, Sigma). Hep-2 and FaDu cells were treated with 5μM, 10μM, and 20μM XCT790 under hypoxic condition which was generated by the Anaerogen compact system for 72

and 48 hours, respectively at 37°C, 5% CO_2 incubator.

RNA isolation and real time-PCR

Total RNA was isolated using Tri Reagent Kit (Sigma-Aldrich), according to the manufacturer's protocol. The concentration and purity of RNA samples were measured using the Implen brand Nano photometer. cDNA was synthesized using The High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) with 1µg of total RNA, and quantitative PCR analyses were performed using Applied Biosystems 7500 instrument (Applied Biosystems). The housekeeping gene β-actin was used as an internal control for normalization. Taqman Gene Expression Assays (HIF1A Hs00153153_m1, HIF2A Hs01026149_m1, SOX2 Hs01053049_s1, NANOG Hs04260366_g1, OCT4 Hs00999632_g1, CD133 Hs01009250_m1, ESRRA Hs01067166_g1, ACTB, Hs99999903_m1) were used to determine gene expressions levels. Thermal cycle parameters were 2 minutes at 50 ° C, 10 minutes at 95 ° C, 15 seconds at 95 ° C and 40 cycles for 1 minute at 60°C. Relative mRNA expression was measured using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

Statistical analyses were performed by using the SPSS software version 17.0. The difference between the expression values of each gene in terms of exposure time to hypoxia was investigated by the Kruskal Wallis test. The difference between XCT790 applications and the expression of genes was investigated by the Kruskal Wallis test. $p < 0.05$ was considered significant.

RESULTS

Changes in gene mRNA levels in Hep-2 cells exposed to hypoxia for 6, 12, 24, 48, 72 hours were shown in Figure 1. There was a significant difference between gene mRNA expressions in terms of exposure time to hypoxia (all genes $p=0.005$, $p<0.05$). Increased mRNA expression levels of genes except HIF-1α were observed after 24h of hypoxia exposure.

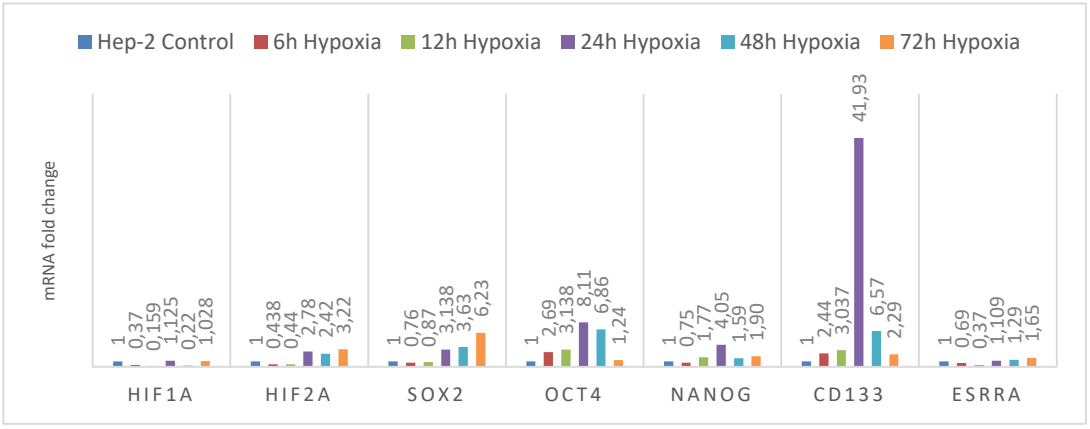

Figure 1. Changes in HIF-1α, HIF-2α, SOX2, OCT4, NANOG, CD133, ESRRA mRNA levels in Hep-2 cells after hypoxia exposure

(HIF-1α: Hypoxia-inducible factor 1-alpha, HIF-2α: Hypoxia-inducible factor 2-alpha, SOX2: SRY (sex determining region Y)-box 2, OCT4: Octamer-binding transcription factor 4 (POU5F1), NANOG: Nanog Homeobox, CD133: Cluster of differentiation133 (PROM1), ESRRA: Estrogen Related Receptor Alpha)

Changes in gene mRNA levels in FaDu cells exposed to hypoxia for 6, 12, 24, 48, 72 hours were shown in Figure 2. There was a significant difference between gene mRNA expressions in terms of exposure time to hypoxia (SOX2 $p=0.006$, other genes $p=0.005$, $p<0.05$). Increased mRNA expression levels of genes except HIF-1α and SOX2 were observed after 12h of hypoxia exposure.

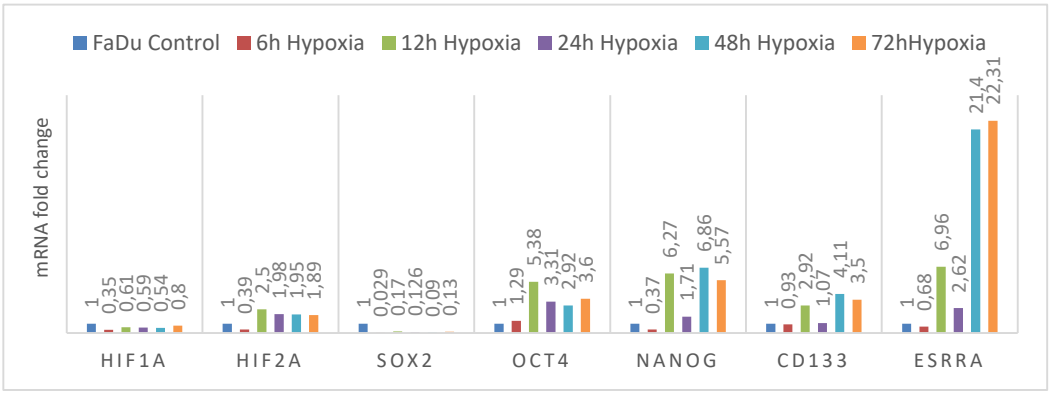

Figure 2. Changes in HIF-1α, HIF-2α, SOX2, OCT4, NANOG, CD133, ESRRA mRNA levels in FaDu cells after hypoxia exposure

(HIF-1α: Hypoxia-inducible factor 1-alpha, HIF-2α: Hypoxia-inducible factor 2-alpha, SOX2: SRY (sex determining region Y)-box 2, OCT4: Octamer-binding transcription factor 4 (POU5F1), NANOG: Nanog Homeobox, CD133: Cluster of differentiation133 (PROM1), ESRRA: Estrogen Related Receptor Alpha)

Hep-2 cells were treated with 5μM, 10μM, and 20μM XCT790 under hypoxic conditions for 72 hours. Significant differences in gene expressions were observed. (HIF-2α p=0.003, other genes p=0.002, p <0.05). In Hep-2 cells exposed to 72h hypoxia, 5μM XCT790 decreased the mRNA expression of genes except CD133. 10μM XCT790 increased mRNA expressions, except HIF-1α. 20μM XCT790 increased mRNA expressions aside from HIF-1α and CD133 (Figure 3).

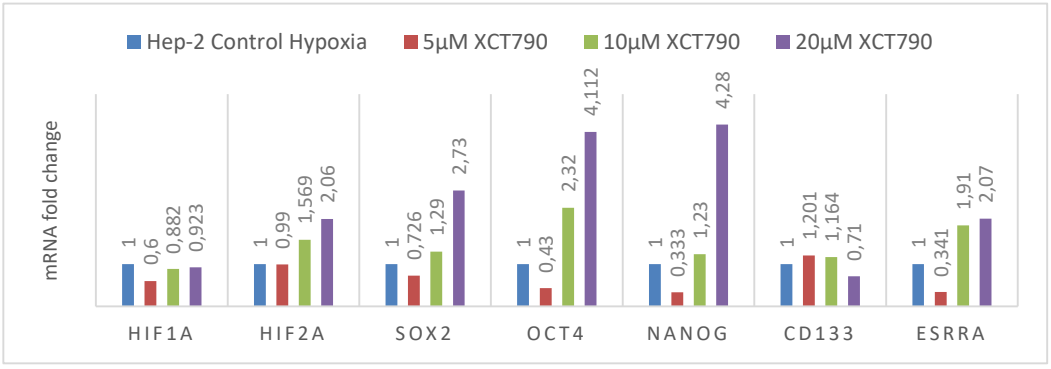

Figure 3. Changes in gene expressions after administration of different doses of XCT790 in Hep-2 Cells

(HIF-1α: Hypoxia-inducible factor 1-alpha, HIF-2α: Hypoxia-inducible factor 2-alpha, SOX2: SRY (sex determining region Y)-box 2, OCT4: Octamer-binding transcription factor 4 (POU5F1), NANOG: Nanog Homeobox, CD133: Cluster of differentiation133 (PROM1), ESRRA: Estrogen Related Receptor Alpha)

FaDu cells were treated with 5μM, 10μM, and 20μM XCT790 under hypoxic conditions for 48 hours. Significant differences in gene expressions were observed (p=0.0001 for all genes, p <0.05). 5μM XCT790 decreased SOX2, OCT4, and CD133 mRNA expressions. 10μM XCT790 reduced only SOX2 mRNA expression and 20μM XCT790 decreased only SOX2 and OCT4 mRNA expressions (Figure 4).

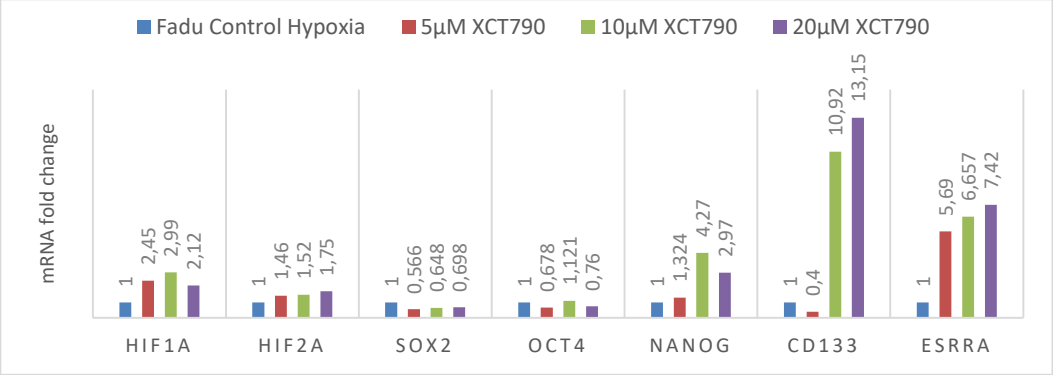

Figure 4. Changes in gene expressions after administration of different doses of XCT790 in FaDu Cells

(HIF-1α: Hypoxia-inducible factor 1-alpha, HIF-2α: Hypoxia-inducible factor 2-alpha, SOX2: SRY (sex determining region Y)-box 2, OCT4: Octamer-binding transcription factor 4 (POU5F1), NANOG: Nanog Homeobox, CD133: Cluster of differentiation133 (PROM1), ESRRA: Estrogen Related Receptor Alpha)

DISCUSSION

Hypoxia is defined as insufficient oxygen supply in organs, tissues or cells. Reduction in oxygen supply may arise from insufficient blood vessel network, abnormal blood vessels, anemia, or increased oxygen consumption because of high cell proliferation. Hypoxia may be physiological or pathological in conditions such as cancer, rheumatoid arthritis, and atherosclerosis. Hypoxic environment is usually provided by $CoCl_2$ solution or hypoxia incubators in mammalian cell cultures[22]. In our study, we created a hypoxic environment by using the "Anaerogen W-Zip Compact" system, which is more affordable and simpler and does not require special gas combinations. In this method, to create hypoxic environment, a package containing ascorbic acid which will reduce oxygen level to 1% within half an hour is placed in a plastic bag. The experimental conditions are checked with the anaerobic indicator that is placed in the bag. Since similar results are achieved when compared with the other methods, there are various theses and publications which this method is used for hypoxia treatments[23-31].

Cancer cells develop adaptation to the hypoxic environment by HIFs, which are the main regulators of hypoxic gene expression and oxygen homeostasis[32]. In our study, when we exposed Hep-2 and FaDu cells to hypoxia for 6-12-24-48 and 72 hours, we found a significant increase in the mRNA expression levels of HIF-2α between the groups in both cell lines. We observed fluctuations in HIF-1a mRNA levels in Hep-2 cells and a decrease in FaDu cells. Although HIF-1α and HIF-2α expressions are usually regulated at the post-translational level, cell culture and case-control studies show that their expression also can be regulated at the mRNA level under hypoxic conditions. Li et al. reported that when glioma stem cells were exposed to hypoxia for varying periods, similar to our findings, they observed no significant change in HIF-1α mRNA but a significant increase in HIF-2α mRNA was detected[33]. In another study investigating the regulation of HIF-1α and HIF-2α in neuroblastoma, in accordance with our study, Hamidian et al. found that hypoxia increased HIF-2α mRNA expression[21]. However, in Zúñiga et al.'s study on lung cancer cells, contrary to our results, they observed that HIF-1α mRNA expression increased, while HIF-2α mRNA expression did not change under hypoxic conditions[34]. In a study with human embryonic stem cell culture, in contrast to our findings, Forristal et al. found no significant difference in physiological and low oxygen levels in terms of HIF-2α mRNA expression, but HIF-2α protein expression showed a significant increase in low oxygen levels[7].

Although it is known that HIF-1α expression usually increases with hypoxia exposure, there are also studies in the literature, similar to ours[35-37]. Chamboredon et al. showed that HIF-1α mRNA levels decreased after 3 hours of hypoxia exposure in

HMEC-1 endothelial cells[35]. Cavadas et al. observed a reduction in HIF-1α mRNA level after 8 hours of hypoxia exposure in HEK293, normal and breast cancer cells[36]. Bartoszewska et al. showed a decline in HIF-1α mRNA level after 2 hours of hypoxia exposure in HUVEC cells[37]. Uchida et al. demonstrated that when A549 cells were exposed to hypoxia for 4, 6, and 12 hours, hypoxia-induced HIF-1α mRNA expression decreased after 4 hours, but HIF-2α mRNA levels continued to increase[38]. These results suggest that prolonged hypoxia regulates the mRNA expressions of HIF-1α and HIF-2α differently.

According to our results, mRNA expression of genes except HIF-1α increased after 24h of hypoxia exposure in Hep-2 cells, and mRNA expression of the genes except HIF-1α and SOX2 elevated after 12h of hypoxia exposure in FaDu cells. Covello et al. and Forristal et al. showed that HIF-2α is an upstream regulator of OCT4[7,39], SOX2 and NANOG[7]. Mathieu et al. reported that HIFs induce embryonic stem cell marker genes in different cancer cell lines[40]. Although we did not investigate protein expressions, some studies in the literature have suggested that HIF-2α protein may have increased embryonic stem cell genes expression by binding to their promoter regions, during hypoxia exposure in Hep-2 and FaDu cells. Many reports have shown that HIF-1α protein expression increases in the early stages of hypoxia exposure, and HIF-2α protein expression increases in prolonged hypoxia[38,41]. However, the decrease of SOX2 mRNA expression in FaDu cells with exposure to hypoxia must need further investigation.

In a study investigating the effect of hypoxia on ovarian cancer cells, Liang et al. found prolonged G0/G1 phase in cells exposed to hypoxia, and the cells expressed higher amounts of CD44, CD133, OCT4, SOX2 in accordance with our study[42]**.** Consistent with our results, in a study conducted on 11 cancer cell lines including prostate, lung, liver, brain, cervix, kidney, colon, and breast tumors, Mathieu et al. found that HIFs induced the expressions of IPSC inducers, SOX2, OCT4, NANOG, cMYC, KLF4, and miR-302 under hypoxic conditions[40].

ERRα contributes to cancer development processes through different pathways in different cancer types[20,21,44-46]. Inhibition of ERRα, via the ERRα agonist XCT790, has been shown to reduce epithelial-mesenchymal transition [47], proliferation[20,48] and induce apoptosis[49] in different cancer cell lines. In our study, we planned to investigate the relationship between ERRα and hypoxia. Adaptation to the hypoxic environment is mediated through hypoxia-inducible factors. In Ao et al.'s study, it was shown that ERRs physically interact with HIFs and stimulate HIF-induced transcription therefore ERRs appear to be essential for HIF's function[18].

Rasbach et al. found that the transcriptional regulation of HIF-2α in skeletal muscle is dependent on the PGC-1α/ERRα complex[43]. In our study, it was observed that mRNA expression of ERRα gene increased in Hep-2 and FaDu cells exposed to hypoxia, similar to the study of Cunningham et al.[19]. In Hamidian et al.'s study, they observed that ERRα mRNA expression did not change with hypoxia exposure, and when they treated cells with the ERRα agonist XCT790 in order to understand the effect of ERRα on HIF-2α transcription in neuroblastoma cells, they detected dose-dependent inhibition of HIF-2α mRNA[21], unlike our results.

In the literature, studies show that XCT790 reduces ERRα protein expression, decreases cell proliferation, and increases apoptosis in different cell lines[48,50-52]. Based on these studies, we treated Hep-2 and FaDu cells with different concentrations of XCT790 to examine the ESC marker gene expression changes, which were increased by hypoxia exposure. When 72-hour hypoxia exposure was used as control, we observed that 5μM XCT790 decreased the mRNA expression of genes, except CD133 in Hep-2 cells. When 48-hour hypoxia exposure was used as control, we found that the mRNA expressions of SOX2 and OCT4 decreased, but the mRNA expressions of other genes increased at the applied XCT790 concentrations in FaDu cells. In this study, we found XCT790 was able to alter gene expression, but we did not detect any dose-related regular changes.

ERRα has been found to bind to the promoter region of metabolic genes associated with cancer development and progression, including glycolysis, TCA cycle, mitochondrial metabolism, OXPHOS, amino acid metabolism, and lipid synthesis and regulates their expression[53]. ERRα regulates energy homeostasis by interacting with PGC-1α and PGC-1β. They control the transcription of the oxidative phosphorylation pathway-related genes[54]. In a study by Wu et al., it was found that XCT790 reduced ERRα and PGC-1α protein expressions and also decreased mitochondrial masses and impaired mitochondrial function in HepG2 and

chemotherapy-resistant R-HepG2 cells. Mitochondrial dysfunction causes the formation of reactive oxygen species (ROS) in the cell and leads to apoptosis with the activation of caspases. In the same study, Wu and colleagues showed that XCT790 increased ROS and caspase 3/7,8,9 activities in both cells and led to apoptosis[55]. In another study with ER-α negative endometrial cancer cell lines, Kokabu et al. observed that XCT790 inhibited cell proliferation by suppressing the Akt/mTOR pathway and induced apoptosis with caspase3/7 activation[56]. Although we did not investigate the apoptotic effect of XCT790, these results suggest that the decrease in the ESC marker gene expressions in our study may also be due to the reduction in cell number.

In conclusion, our study revealed that hypoxia exposure changed the HIFs and ESC marker gene expressions in Hep-2 and FaDu cells and the ESRRA inverse agonist XCT790 could affect gene expressions that changed as a result of hypoxia. This is the first study which ESRRA mRNA expression, an important cofactor in the HIF-mediated hypoxic response, has been studied in laryngeal and pharyngeal cancer cells. Our results suggest that although ESRRA is a cofactor of the hypoxic response, its inhibition alone may not be sufficient to abolish the hypoxic response. The limitations of this study are the lack of protein data and the inability to investigate apoptotic gene expressions. Protein data is needed to reveal the precise mechanisms of ESRRA and hypoxia association in head and neck cancers. Also, since XCT790 is an apoptosis inducer, it is planned to investigate apoptosis-related genes to better understand the effect of XCT790 on head and neck cancers.

Yazar Katkıları: Çalışma konsepti/Tasarımı: NSI, DA, MBY; Veri toplama: NSI, DA, MBY, HÖ; Veri analizi ve yorumlama: NSI, DA, MBY, HÖ, NK; Yazı taslağı: NSI, DA; İçeriğin eleştirel incelenmesi: NSI, DA, MBY, HÖ, NK; Son onay ve sorumluluk: NSI, HÖ, MBY, NK, DA; Teknik ve malzeme desteği: DA, MBY; Süpervizyon: NSI, DA, MBY, HÖ; Fon sağlama (mevcut ise): yok.
Etik Onay: Bu çalışmada insan larinks (Hep-2) ve farinks (FaDu) karsinom hücreleri kullanılmıştır. Hücreler ticari olarak satın alındığı için etik kurul onayı gerekmemektedir.
Hakem Değerlendirmesi: Dış bağımsız.
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Yazarın Notu: *Bu makale birinci yazarın doktora tezinden üretilmiştir.

Author Contributions: Concept/Design : NSI, DA, MBY; Data acquisition: NSI, DA, MBY, HÖ; Data analysis and interpretation: NSI, DA, MBY, HÖ, NK; Drafting manuscript: NSI, DA; Critical revision of manuscript: NSI, DA, MBY, HÖ, NK; Final approval and accountability: NSI, HÖ, MBY, NK, DA; Technical or material support: DA, MBY; Supervision: NSI, DA, MBY, HÖ; Securing funding (if available): n/a.

Ethical Approval: In this study, human larynx (Hep-2) and pharynx (FaDU) carcinoma cells were used. Approval of the ethics committee is not required because the cells are purchased commercially.
Peer-review: Externally peer-reviewed.
Conflict of Interest: None of the authors have any conflict of interest related with this study.
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