SHORT COMMUNICATION

**Functional activity of aryl hydrocarbon receptor in human osteosarcoma cell cultures**

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Osteosarcoma is the most prevalent bone malignant tumor with a high mortality rate among children and adolescents. The aryl hydrocarbon receptor (AHR) is a ligand-dependent transcription factor associated with xenobiotic detoxification and carcinogenesis. It is known that some AHR ligands are included in the composition of drugs used in cancer therapy. However there are few works devoted to the study of their effect on osteosarcoma cells. In this work, three primary cell cultures were obtained from biopsy material of malignant bone tumors of human osteosarcoma. It was shown that the aryl-hydrocarbon receptor is functionally active in all cultures, but the target genes were induced differently by ligand treatment within the same cell culture.

**Keywords**: *aryl hydrocarbon receptor AHR,* *cytochrome P450 family CYP1, osteosarcoma, indirubin, indol-3-carbinol, cell culture*

**Running title:** Functional activity of AHR in osteosarcoma cell cultures

**Osteosarcoma (also called osteogenic sarcoma) is the most common primary bone cancer. It starts in the bone cells. Osteosarcoma has been associated with rapid progression, early metastasis, a high risk of recurrence following surgery, and poor prognosis. It most often occurs in young people. Incidence peaks during the period of rapid growth: at age 10–14 in females and at age 15–19 in males [1]. Despite numerous studies aimed at optimizing the systemic treatment regimens for sarcomas [2–4], the treatment efficacy of these malignant tumors remains low. The accumulated data indicate the need to investigate the factors that affect the disease prognosis and outcomes of chemotherapy as well as to search for alternative treatment methods for osteosarcoma.**

**The Aryl Hydrocarbon Receptor (AHR) is a ligand-dependent transcription factor whose functions are related to xenobiotic detoxification and carcinogenesis. A wide spectrum of compounds that function as AHR ligands are currently known, including food substances, natural and synthetic flavonoids, and pharmaceuticals, including those used in anticancer therapy [5–7].**

**Before ligand binding, AHR is retained in the cytoplasm in a complex with an Hsp90 (Heat Shock Protein 90) dimer, the p23 cochaperone, and the AIP (AHR Interacting Protein) protein. Binding to its ligands leads to dissociation of the complex, and AHR translocates into the nucleus, where it binds to the Aryl Hydrocarbon Receptor Nuclear Translocator (AHR–ARNT) [8]. The formed AHR : ARNT heterodimer interacts with XRE (Xenobiotic Response Element) regulatory elements of the responsive genes, which upregulates their transcription [9–10].**

**The most studied AHR targets are the genes encoding xenobiotic-metabolizing enzymes, such as cytochrome P450 (cytochrome P450, CYP). In many tumor cells, the changes in the ratio of different CYP isoforms and their inducibility are known to be different to the changes occurring in noncancerous cells, and this can strongly affect the treatment efficacy with antitumor drugs.**

**AHR is regarded as a potential target for anticancer therapy [5–7]. Despite many studies focused on the study of AHR activity in different types of cancer, few investigations into its role in bone malignancies, including osteogenic sarcomas, are available. The aim of our work was to study the functional activity of AHR in primary cultures of human osteogenic sarcoma cells after exposure to its exogenous ligands.**

**Materials and Methods**

**Preparation of primary osteosarcoma cell cultures.** Tumor tissue specimens were obtained in 2018 from the biopsy material of patients diagnosed with conventional osteosarcoma, the osteoblastic histological type. The patients included males and females aged 14–16 years. Chemotherapy was not performed before collection of the biopsies. Tumor specimens were transported to the laboratory within 2–3 h in a serum-free DMEM medium (Dulbecco’s Modified Eagle Medium, Paneco, Russia) supplemented with 100 μg/mL of gentamicin (Microgen, Russia). Afterwards, the tissue was disaggregated mechanically or enzymatically (trypsin-EDTA 0.25% solution, Paneco, Russia) to produce a suspension of cells, which was placed into culture flasks (Corning Costar, United States) containing DMEM/F12 medium (Gibco, United Kingdom) supplemented with 10% fetal bovine serum (PAA Laboratories, Austria) and gentamicin (50 μg/mL).

***Cell lines and cultivation conditions.*** Human fetal kidney cell lines HEK293 and mesenchymal stem cells MSC were cultured in DMEM/F12 medium (Gibco, United Kingdom) supplemented with 10% fetal bovine serum (PAA Laboratories, Austria) and gentamicin (50 μg/mL).

***Treatment of cells with ligands.*** Primary cultures of osteosarcoma cells were seeded into six-well plates. In 24 h, ligands were added into the culture medium: indirubin at a final concentration of 100 nM (Sigma-Aldrich, United States) and indole-3-carbinol (100 μM) (Mirax Biopharma, Russia). Stock solutions of the ligands were prepared using DMSO (dimethyl sulfoxide; Paneco, Russia). The same amount of DMSO was added to the control cell sample. The cells were incubated with the ligands for 24 h.

***Extraction of RNA, RT-PCR, and real time PCR.*** Total RNA was extracted using RNAzol RT reagent (Sigma-Aldrich, United States) according to the manufacturer’s guidelines. The quality of the isolated RNA was assessed by electrophoresis in 1% agarose gel containing 0.01% ethidium bromide. The RNA concentration was measured using the NanoDrop 8000 spectrophotometer (Thermo Scientific, United States). Reverse transcription was performed using the MMLV RT Kit (Eurogen, Russia), and real-time PCR was conducted using the qPCRmix-HS SYBR+HighRox reagent kit (Eurogen, Russia). The reaction was performed in the ABI Prism 7500 Sequence Detection System (Applied BioSystems, United States). Amplification conditions: 95° for 5 min then 40 cycles (95° for 15 s, 60° for 15 s, 72° for 30 s). The reference genes were GAPDH and HPRT1. The gene expression level was calculated using the 2-ΔΔСt method. The sequences of primer pairs are as follows. For the GAPDH gene: forward–TGCACCACCAACTGCTTAGC, reverse–GGCATGGACTGTGGTCATGAG; for the HPRT1 gene: forward–TGAGGATTTGGAAAGGGTGT, reverse–GAGCACACAGAGGGCTACAA; for the CYP1A1 gene: forward–GATTGAGCACTGTCAGGAGAAGC, reverse–CCAAAGAGGTCCAAGACGATGTTA; for the CYP1A2 gene: forward–ATCCTGGAGACCTTCCGACACT, reverse–GATGTAGAAGCCATTCAGCGTTGTG; for the CYP1B gene: forward–CTCAACCGCAACTTCAGCAACTTC, reverse–AGAGAGGATAAAGGCGTCCATCAT. Each specimen was analyzed in three repeats. Statistical significance of the differences between the specimens was estimated using the REST software (Qiagen, United States) [16]. A P-value of less than 0.05 was considered significant.

***Western blotting.***Total cell protein was lysed in a Lammley buffer (10% SDS, 50 mM Tris-HCl pH 6.8, 25% glycerol, 0.05% bromophenol blue, and 6% 2-mercaptoethanol). Protein mixture was separated by SDS polyacrylamide gel electrophoresis using the Mini Trans-Blot cell (Bio-Rad, United States) as recommended by the manufacturer’s protocol; proteins were transferred to a Hybond ECL membrane (Sigma Aldrich, United States). The membrane was incubated in a TBS-T buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, and 0.1% Tween 20) containing 2% BSA for 1 h and then incubated with primary antibodies at 4°C overnight (antibodies to AHR were D5S6H, Cell Signaling Technology, United States, 1 : 7000; antibodies to GAPDH were ab9385, Abcam, United Kingdom, 1 : 10000). Subsequently, the membrane was washed with TBS-T buffer thrice within 20 min each time and incubated with secondary antibodies (С1313, Santa Cruz, United States, 1 : 12000) for 2 h at room temperature. Immunocomplexes were detected using the ECL Advance Western Blotting Detection Kit (Sigma Aldrich, United States).

**Results and Discussion**

Three primary cultures were obtained from biopsy material of human malignant bone tumor osteosarcoma (O.src 25/16, O.src 17/18, and O.src 20/18). Osteosarcoma cultures had an increased content of AHR protein compared to noncancerous cells HEK293 and MSC (Fig. 1).

In addition, we compared the initial CYP1 mRNA level in osteosarcoma cell cultures without AHR activation by ligands. The CYP1A1 gene showed the highest expression level in O.src20/18, the CYP1A2 gene in O.src17/18, and the CYP1B gene in culture O.src25/16 (Fig. 2). The functional activity of AHR was assessed from changes in the expression of cytochrome P450 family 1 genes (CYP1A1, CYP1A2, and CYP1B) after exposure of the cells to AHR known exogenous ligands, i.e., indirubin and indole-3-carbinol.

In our comparative experiments, it was shown that the aryl hydrocarbon receptor was functionally active in most cases as a ligand-dependent transcription factor of the CYP family target genes in primary osteosarcoma cell cultures. Interestingly, the target genes of the cytochrome P450 family CYP1, which participate in metabolism, were induced differently by ligand treatment within the same cell culture. In addition, the intensity of activation of the same gene by different ligands was different.

It is possible that the effects of AHR activation in osteosarcoma cell cultures depend on other factors, such as the epigenetic status of its target genes and existence of unknown endogenous ligands, which is a subject that demands further investigation.

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**Figure Captions**

**Fig. 1.** (**A**) Immunoblotting of AHR protein in the obtained osteosarcoma cell cultures O.src20/18, O.src17/18, O.src25/16 and (**B**) in noncancerous cell lines (HEK293, MSC). GAPDH was the reference protein.

**Fig. 2.** Constitutive CYP gene expression in osteosarcoma cell cultures without activation by AHR ligands. (**A**–**C**): mRNA level were aligned relative to cell line O.src17/18. The Y axis represents R = 2ΔСt, i.e., the ratio of mRNA quantity of the target gene to the mRNA quantity of housekeeping genes GAPDH and HPRT1.