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ORIGINAL PAPER

Involvement of Aryl Hydrocarbon Receptor in L-Kynurenine-Mediated Parathyroid Hormone–Related Peptide Expression

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Abstract

Parathyroid hormone-related peptide (PTHrP), produced by specific cancers such as lung cancer, profoundly influences the formation of bone metastatic lesions via the "vicious cycle" of tumor growth and bone resorption. The changes in gene expression regulated by the abnormal microenvironment components play key roles in maintaining the biological characteristics of cells, such as the organotropism of cancer metastasis. A recent study has shown that L-kynurenine (L-Kyn), one of microenvironment components, induced a substantial increase in the metastasis of lung cancer cells. What remains unclear, however, is the linkage between L-Kyn and bone metastatic lesions. In the present paper, we found that a significant upregulation of PTHrP expression was detected when 95D cells, a lung cancer cell line, were incubated with 50 μM of L-Kyn. Meanwhile, L-Kyn (50/100 μM) strongly strengthened aryl hydrocarbon receptor (Ahr) expression. Additionally, L-Kyn (50 μM) increased the expression of the nuclear translocation of Ahr and cytochrome P450 1A1. Most importantly, the L-Kyn-induced upregulation of migration was significantly reduced when cells were co-incubated with $siRNA_{Ahr}$. Notably, the L-Kyn-mediated increase in PTHrP was also substantially attenuated upon $siRNA_{Ahr}$ treatment in 95D cells. These results suggest that Ahr is involved in the L-Kyn-induced enhancement of PTHrP expression.

Keywords L-kynurenine · Bone metastatic lesions · Parathyroid hormone-related peptide · Aryl hydrocarbon receptor

Introduction

The metastatic spread of tumor cells to vital organs results in mortality and is the second leading cause of death in the developed world [1, 2]. Interestingly, certain types of cancer cells metastasize predominantly to specific organs. Bone is the preferential site of metastasis for breast, prostate, and lung tumors. However, the molecular mechanisms that govern the spread of cancer cells to the bone remains unclear, although parathyroid hormone-related peptide (PTHrP), a 139- to 173-amino acid protein with N-terminal homology to the parathyroid hormone, is considered to be the principal molecule in the formation of metastatic lesions in the bone via the "vicious cycle" of tumor growth and bone resorption [3]. Previous studies have shown that the metastatic process is complex and often related to gene expression abnormity controlled by the components of the tumor microenvironment [4–7].

L-kynurenine (L-Kyn), one of microenvironment components, is the metabolite generated by indoleamine 2,3-dioxygenase (IDO), which is a key enzyme catalyzing the first and rate-limiting steps along the kynurenine pathway of tryptophan metabolism outside the liver. In non-small cell lung cancer (NSCLC), augmented tryptophan catabolism, which results in higher L-Kyn serum concentration, is linked to a more advanced stage at diagnosis, poorer clinical prognosis, and a lesser likelihood of response to chemotherapy [8–10], which indicates that L-Kyn may play key roles in the physiological and biochemical processes of cancer. Specifically, L-Kyn not only inhibits innate immune responses through mechanisms such as inducing the apoptosis of natural killer cells through the generation of reactive oxygen species [11], but

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also attenuates cell immune responses through the activation of the aryl hydrocarbon receptor (Ahr) [12] and humoral immune response by enhancing the expression of key transcription factors that negatively regulate B cell terminal differentiation [13, 14]. These results indicate that L-Kyn promotes the survival of cancer cells by inhibiting the immune system. Additionally, L-Kyn also significantly promotes the metastasis of lung cancer cells [15]. What remains unclear, however, is the linkage between L-Kyn and the formation of bone metastatic lesions.

Ahr is activated by xenobiotics, notably dioxin, as well as by exogenous and endogenous metabolites (such as L-Kyn), and then transferred into the nucleus. Next, Ahr and Ahr nuclear translocator (ARNT) form a heterodimeric basic helix-loop-helix-Per-ARNT-Sim (bHLH-PAS) domain [16], which is capable of binding to dioxin-responsive elements (DRE) located in target DNA, leading to increased expression of downstream genes, including xenobiotic metabolizing enzymes such as cytochrome P450 (CYP) 1 family members: *CYP1A1* and *CYP1B1* [17]. Therefore, *CYP1A1* is considered a biomarker of Ahr activation, and Ahr plays key roles in many physiological and biochemical processes [17].

In the current paper, we found that L-Kyn significantly increased PTHrP expression in lung cancer cells. Moreover, the L-Kyn-mediated enhancement of PTHrP was substantially attenuated upon $siRNA_{Ahr}$ treatment. These results suggest that L-Kyn plays a role in the metastasis of tumors to the bone and that Ahr is involved in the upregulation of PTHrP expression induced by L-Kyn.

Materials and Methods

Cell Culture and Transfection

95D cells, a human lung cancer cell line, were obtained from Beijing Chuanglian North Carolina Biotechnology Research Institute and cultured in 1640 medium (HyClone) supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 U/ml penicillin.

All transfection experiments in this paper were conducted by the GenMute™ siRNA Transfection Reagent (SL100568, SignaGen), according to the manufacturer's recommendations.

Real-Time PCR Analysis

Total RNA was isolated from 95D cells by TRIzol and reverse-transcribed to cDNA by EasyScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech). Target mRNA was quantified using real-time PCR reactions with TransStart Tip Green qPCR SuperMix (TransGen Biotech), and GAPDH was served as an internal control. The conditions for real-time PCR were 40 cycles of denaturation (95 °C/15 s), annealing (56.5 °C/30s), and extension (72 °C/15 s). The primers used were listed in Table 1.

Western Blot Analysis

Total cellular proteins were harvested in RIPA lysis buffer from 95D cells. The samples were subjected to SDS-PAGE separation, and proteins were transferred to PVDF membranes. Next, the membranes were incubated with the primary antibodies against PTHrP (ab125700, Abcam), Ahr (BA2013, BOSTER), CYP1A1 (PB0573, BOSTER), and β-actin (ab8226, Abcam), respectively. Finally, immunoreactivity was detected by incubation with HRP-labeled anti-rabbit/mouse IgG antibody (ab6721, Abcam; sc-2005, Santa Cruz Biotechnology), respectively. Quantitation of protein expression was done by densitometry and normalized with loading control (β-actin).

Immunofluorescence Assay

95D cells were administered with L-Kyn (50 μM) or without and fixed with 4% formaldehyde for 20 min. Next, cells were permeabilized by 0.1% Triton X-100 solution for 10 min, and incubated with goat serum for 20 min. Anti-Ahr rabbit polyclonal antibody was added and incubated at room temperature for 2 h. Goat anti-rabbit IgG H&L (FITC) (ab6717, Abcam) was subsequently added and incubated for another 1 h. Cell nuclei were stained with DAPI solution. Finally, the fluorescent image was observed under a fluorescence microscope.

Migration Assay

Migration assays were performed by Transwell with 8-μM pore filter inserts. 95D cells (1×10^4/well) were seeded in the upper chamber. The lower chambers were filled with 1640 medium containing 50 μM L-Kyn or without. When the treatment ended, the inserts were removed, and the cells

Table 1 Sequences of primers used in this study

Gene name		Sequences (5′-3′)
PTHrP	Forward	CAA CCA GCC CAC CAG AGG A
	Reverse	GGC GGC TGA GAC CCT CCA
Ahr	Forward	CAA ATC CTT CCA AGC GGC ATA
	Reverse	CGC TGA GCC TAA GAA CTG AAA G
CYP1A1	Forward	TCG GCC ACG GAG TTT CTT C
	Reverse	GGT CAG CAT GTG CCC AAT CA
GAPDH	Forward	TGT TGC CAT CAA TGA CCC CTT
	Reverse	CTC CAC GAC GTA CTC AGC G
$siRNA_{Ahr}$[18]	Forward	CGG AUG AAA UCC UGA CGU Att
	Reverse	UAC GUC AGG AUU UCA UCA Gtt

that migrated to the lower chamber were fixed. Finally, the cells were stained with 0.1% Crystal Violet, and captured by a microscope (× 40).

Statistical Analysis

All experiments were performed in triplicates, and data were expressed as the mean ± standard error of mean. Statistical analyses were conducted using the professionally statistical computer software, SPSS. Differences between groups were determined using a *t* test. $P < 0.05$ was considered statistically significant.

Results

L-Kyn Promotes PTHrP Protein Expression

To uncover the linkage between L-Kyn and bone metastasis in lung cancer, we analyzed the effects of L-Kyn on PTHrP, which is produced by cancer cells in bone metastatic lesions. As shown in Fig. 1 a, PTHrP expression at the transcription level in the 50 μM L-Kyn treatment group was significantly higher than that in the control group in 95D cells ($P < 0.05$). However, no substantial differences at mRNA level between L-Kyn (30/100/200/500 μM) treatment groups and control group were detected (Fig. 1a). In addition, we also found that L-Kyn (50/100 μM) markedly upregulated the level of PTHrP protein compared to the control group ($P < 0.05$ and $P < 0.05$, respectively; Fig. 1b). Surprisingly, there was also no significant alteration in PTHrP protein expression when incubated with selected doses of L-Kyn (30/200/500 μM) in 95D cells (Fig. 1b). Although the results, no statistically significant, were out of our expect, these results ($P < 0.05$) still revealed that L-Kyn promoted PTHrP expression in 95D cells.

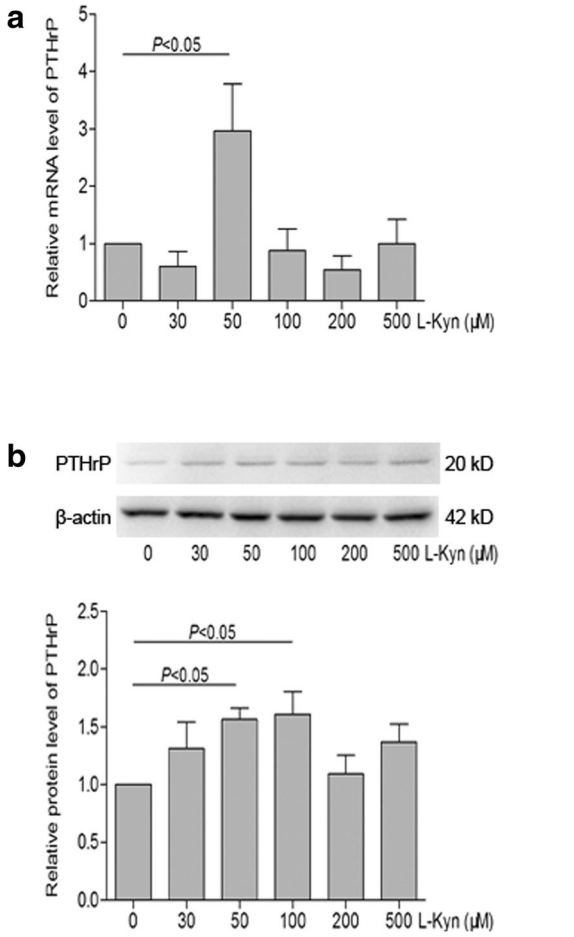

Fig. 1 Effects of L-Kyn on PTHrP expression. 95D cells were cultured and administered with indicated concentrations of L-Kyn. Next, total RNA was extracted and reversely transcribed into complementary DNA. Lastly, real-time PCR was performed to analyze the PTHrP mRNA level (**a**), and western blot was conducted to assay the PTHrP protein expression (**b**)

Upregulation of the Expression and Activity of Ahr by L-Kyn in 95D Cells

L-Kyn is the physiological ligand of Ahr, and Ahr has key roles in many physiological and biochemical processes [17]. It has been reported that Ahr has both pro- and anti-tumor effects in prostate cancer and breast cancer, both tumors with frequent bone metastasis; these effects are context dependent [19, 20]. Therefore, we measured the effects of L-Kyn on Ahr expression. The results show that L-Kyn (30/200/500 μM) failed to induce substantial changes in Ahr expression at the mRNA and protein levels compared to the control group (Fig. 2a, b); however, Ahr was significantly upregulated at the mRNA and protein levels when administered 50 μM ($P < 0.05$ and $P < 0.05$, respectively; Fig. 2a, b) and 100 μM of L-Kyn ($P < 0.05$ and $P < 0.05$, respectively; Fig. 2a, b). Additionally, the nuclear translocation of Ahr was strengthened in 95D cells treated with L-Kyn (50 μM) (Fig. 2c). Meanwhile, significant increases in the expression of CYP1A1, a molecular marker of activated Ahr, were detected at the transcription and translation levels when incubated with 50 μM of L-Kyn ($P < 0.01$ and $P < 0.05$, respectively; Fig. 2d, e). These results indicate that L-Kyn not only activated Ahr, but also upregulated Ahr expression in 95D cells.

Reduction of the L-Kyn-Induced Increase in Cellular Migration by $siRNA_{Ahr}$ Treatment in 95D Cells

In order to explore the effect of Ahr on the metastasis of 95D cells, a Transwell assay was performed to test the effect of Ahr on migration. First, we detected the interference efficiency of $siRNA_{Ahr}$ targeting Ahr. As shown in Fig. 3 a and b, Ahr mRNA and protein expression were strongly reduced by $siRNA_{Ahr}$ treatment ($P < 0.001$ and $P < 0.05$, respectively); as expected, decreasing Ahr significantly attenuated the migration of 95D cells ($P < 0.05$; Fig. 3c). However, the number

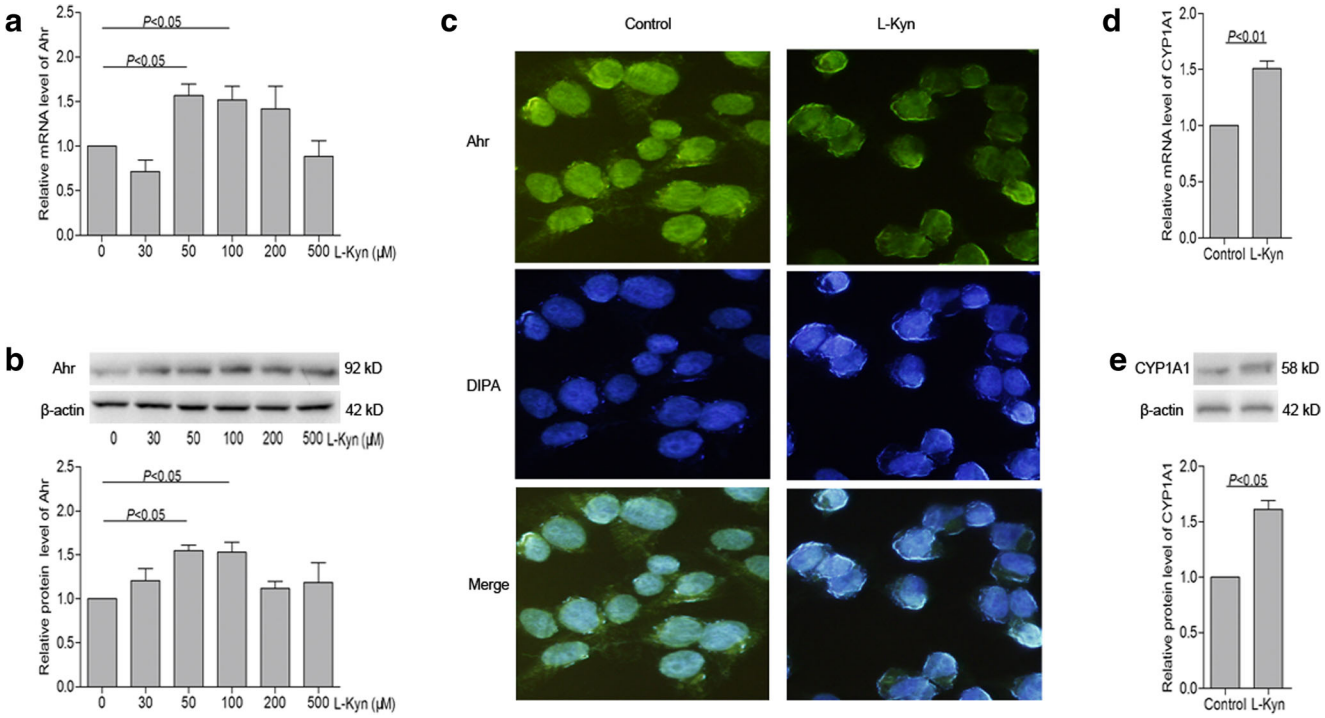

Fig. 2 Effects of L-Kyn on Ahr in 95D cells. 95D cells were cultured and administered with indicated doses of L-Kyn. Real-time PCR was performed to analyze the Ahr (**a**) and CYP1A1 (**d**) mRNA level, and western blot was conducted to assay the Ahr (**b**) and CYP1A1 (**e**) protein expression. The effect of L-Kyn on the nuclear transfer of Ahr was examined by immunofluorescence (**c**)

of cells that migrated to the lower chamber in the L-Kyn (50 μM) treatment group were markedly higher than that in the control group ($P < 0.01$; Fig. 3c). Furthermore, the $siRNA_{Ahr}$-mediated downregulation of cellular migration was substantially reversed upon L-Kyn (50 μM) treatment ($P < 0.01$; Fig. 3c). Most importantly, L-Kyn-induced increase (50 μM) was reduced when the cells were co-incubated with $siRNA_{Ahr}$ ($P < 0.05$; Fig. 3c). These data demonstrate that reducing Ahr inhibited the L-Kyn-mediated upregulation of 95D cell migration.

Attenuation of the L-Kyn-Mediated Increase in PTHrP Expression by $siRNA_{Ahr}$ Treatment in 95D Cells

Next, we examined the effect of Ahr on PTHrP expression and found that reducing Ahr significantly downregulated PTHrP expression at the mRNA and protein levels ($P < 0.05$ and $P < 0.05$, respectively; Fig. 4a, b). Additionally, the attenuation of PTHrP mRNA and protein induced by $siRNA_{Ahr}$ was completely reversed in the presence of 50 μM of L-Kyn ($P < 0.01$ and $P < 0.01$, respectively; Fig. 4a, b). Notably, the L-Kyn-mediated enhancement of PTHrP expression at the transcriptional and translational levels were strongly decreased after $siRNA_{Ahr}$ treatment ($P < 0.05$ and $P < 0.05$, respectively; Fig. 4a, b). These results strongly suggest that Ahr is involved in the L-Kyn-induced increase in PTHrP expression in 95D cells.

Discussion

Most malignant tumors, particularly breast, prostate, and lung primary tumors, preferentially metastasize to the bone [3]. The metastatic organotropism is perfectly explained by the "seed vs soil" hypothesis, which suggests that the local microenvironment of the target organs ("soil") provides an appropriate environment for tumor ("seed") colonization and growth [21]. Once cancer cells, particularly tumor cells that constitutively express PTHrP, reach the skeleton, the produced PTHrP disrupts bone homeostasis, which depends on the dynamic equilibrium between osteoblasts and osteoclasts and the factors that mediate the crosstalk between them. In brief, PTHrP, which has a profound effect on the activities and survival of tumor cells, is beneficial to osteoclastic bone resorption [3], because it stimulates osteoblasts to increase the expression of receptor activator of nuclear factor κB ligand (RANKL). The binding of RANKL to the RANK expressed in premature osteoclasts promotes the differentiation and maturation of osteoclasts, eventually leading to the initiation of bone resorption [22]. Once bone resorption is activated by mature osteoclasts, a large amount of transforming growth factor-β (TGF-β) is released in the bone matrix. TGF-β binds to the TGF-β receptor expressed in cancer cells to stimulate PTHrP production [23]. Thus, TGF-β released by resorbed bone and PTHrP generated by cancer cells consist of the "vicious cycle" [23], which leads to the formation of bone metastatic lesions.

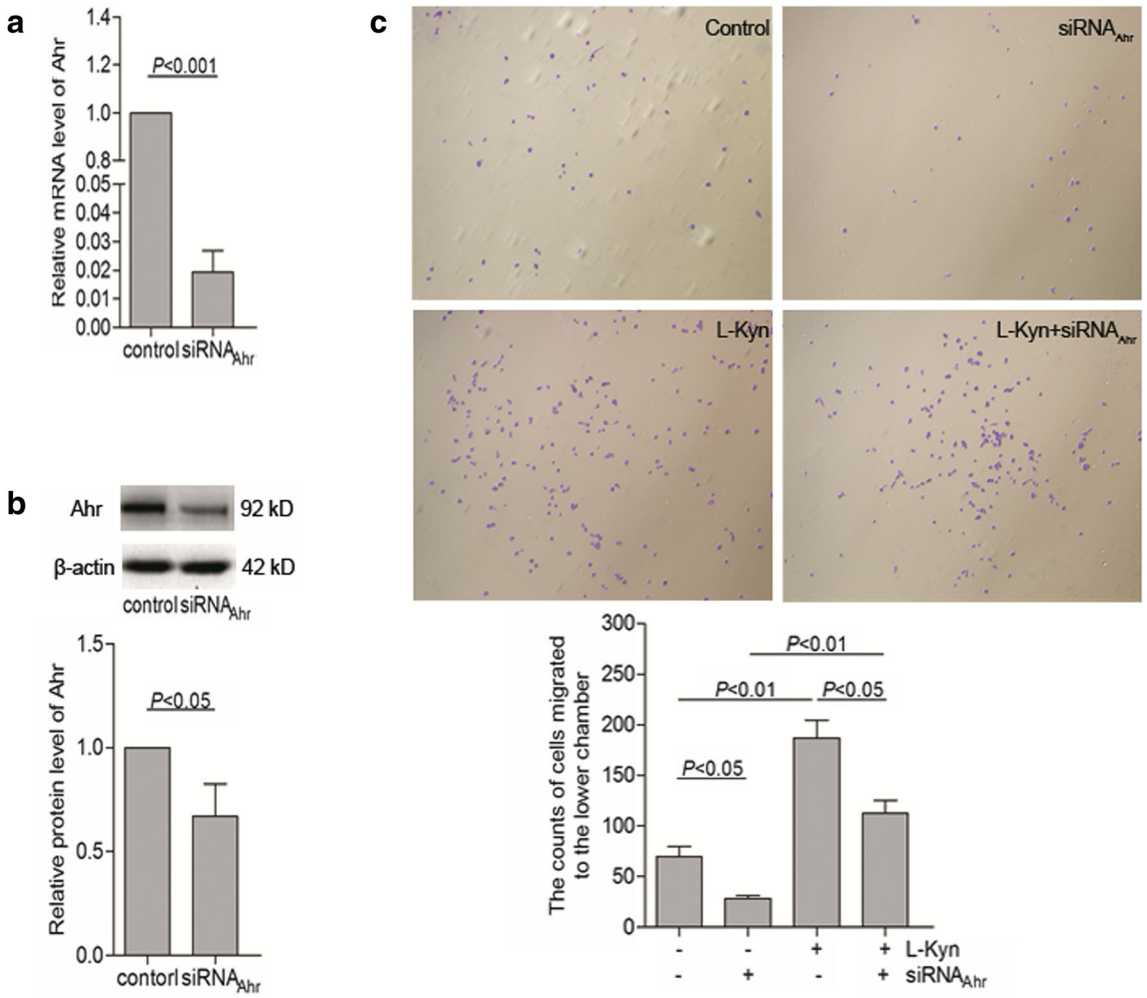

Fig. 3 Effects of Ahr on migration in 95D cells. 95D cells were seeded at 10^4/well and treated with indicated way. Real-time PCR and western blot was performed to analyze the Ahr mRNA level (**a**) and Ahr protein expression (**b**), respectively. The migration of 95D cells was assayed by Transwell (**c**)

However, a question remains: how is constitutive PTHrP expression maintained in cancer cells?

It is generally known that gene expression regulated by the abnormal microenvironment components plays a key role in maintaining the biological characteristics of cells. It has been demonstrated that the activity of IDO is strongly upregulated in lung cancer, leading to an increase in L-Kyn concentration in the tumor microenvironment [24]. A recent study has confirmed that the L-Kyn content in the serum of patients with NSCLC is positively linked to poor prognosis [10]. Meanwhile, metastases are considered the primary cause of death induced by cancer; therefore, these key points may indicate that L-Kyn is closely related to the metastasis of cancer cells. Moreover, previous data has shown that L-Kyn promoted the metastasis of lung cancer cells through different pathways [15]. What remains unclear, however, is the linkage between L-Kyn and bone metastatic lesions. In the current study, although no significant changes in PTHrP expression at the mRNA and protein levels were detected when incubated with L-Kyn (30/200/500 μM) in 95D cells, 50 μM of L-Kyn induced a significant increase in PTHrP expression at the mRNA and protein levels. Interestingly, although L-Kyn (100 μM) failed to induce a substantial change in PTHrP mRNA expression, treatment at this concentration strongly strengthened the expression of the PTHrP protein. This may be explained in the following manner. L-Kyn induced DRE-luciferase activity in glioma cells with a half-maximal response concentration (EC50) of 36.6 μM [17]. Therefore, we speculate that 30 μM of L-Kyn failed to cause significant PTHrP changes because of the low concentration. Furthermore, when the concentration of L-Kyn is higher than 100 μM, the increase in DRE-luciferase activity reaches a relative plateau stage [17], which is partly in accordance with the "tide" model in which the responses induced by stimulants are adjusted and return to baseline due to the tight feedback between co-stimulatory factors and co-inhibitory factors [25]. Thus, the phenomenon that higher concentrations of L-Kyn fail to cause significant differences in PTHrP expression may be attributed to co-inhibitory factors that play a role in the effects of L-Kyn (200/500 μM) on PTHrP expression.

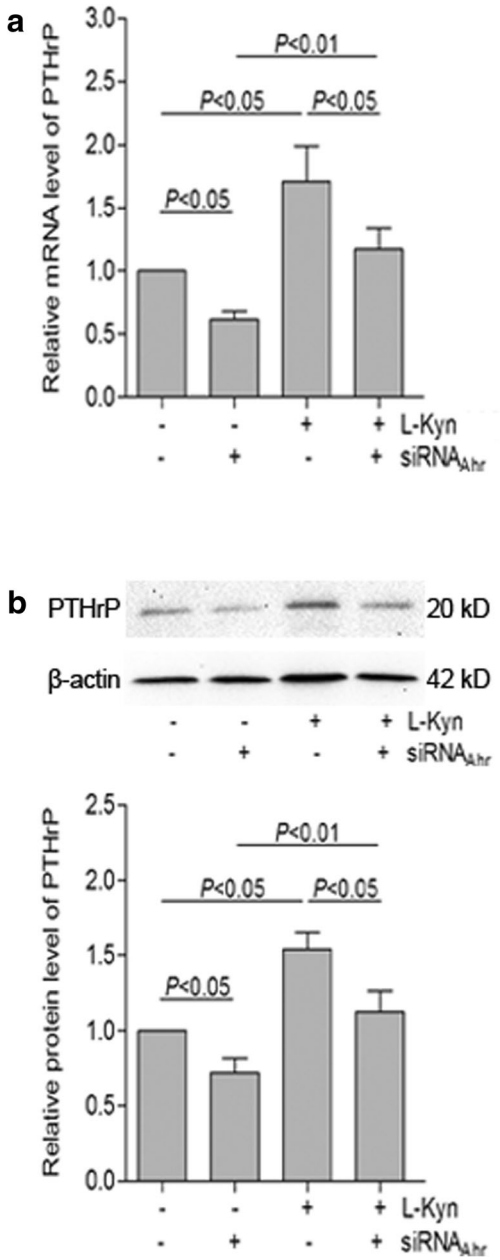

Fig. 4 Effects of Ahr on L-Kyn-mediated the increase of PTHrP in 95D cells. 95D cells were cultured and treated with indicated way. Real-time PCR and western blot was conducted to examine the PTHrP mRNA level (**a**) and PTHrP protein expression (**b**), respectively

Additionally, it is well known that PTHrP expression is also controlled by miRNAs, which are epigenetic regulators. miRNAs can only alter protein expression in mammalian cells when there is partial complementarity between the base sequences of the miRNA and the target gene. Therefore, the differences in PTHrP mRNA and protein expression induced by 100 μM of L-Kyn may result from the regulation of miRNAs. Most importantly, these positive results suggest that L-Kyn maintains constitutive PTHrP expression in lung tumors, finally leading to bone metastasis under appropriate conditions. Next, the mechanism of how L-Kyn controls PTHrP expression was explored.

It has been demonstrated that Ahr plays a central role in L-Kyn-mediated physiological and biochemical processes [17]. In the present study, we found that L-Kyn (50 μM) not only activated Ahr, which was in accordance with the results that Ahr is the physiological receptor of L-Kyn [17], but also significantly enhanced Ahr expression at the transcription and translation levels, which suggested that L-Kyn might play two roles in regulating Ahr expression from a qualitative and quantitative level. Previously, it was shown that the activation of the L-Kyn pathway or increasing L-Kyn concentration induces a significant upregulation of interleukin 6 (IL6) levels in the cerebrospinal fluid of patients with chronic schizophrenia [26]. L-Kyn (50 μM) also significantly promoted IL6 expression in 95D cells (Supplementary Files). Ahr expression is positively correlated with the expression of several genes (IL1B, IL6, tumor necrosis factor [TNF], IL8, and C-X-C chemotype receptor 4 [CXCR4]) [27]; thus, IL6 may be the underlying mechanism in the L-Kyn-induced increase in Ahr expression. Next, we found that Ahr not only promoted the migration of 95D cells, but also strengthened the L-Kyn-mediated increase in migration. This is in accordance with the findings that L-Kyn promotes the formation of the E-cadherin/Ahr/S-phase kinase-associated protein 2 complex and induces the degradation of E-cadherin to increase the migration of tumor cells [28], as well as the finding that Ahr activation by endogenous ligands induces an increase in breast cancer cell migration [29]. However, whether Ahr is involved in the L-Kyn-mediated upregulation of PTHrP expression remains unclear.

Estrogen, androgen, and smoking are closely related to the manifestation of breast, prostate, and lung cancer, respectively. It has been reported that estrogen is the physiological ligand of Ahr [30] that androgen can directly interact with Ahr in the nucleus [19] and that cigarette components can activate Ahr and upregulate CYP1A1 and CYP1B1 expression [31]. These results suggest that Ahr maybe play key roles in bone metastasis due to the high metastatic frequency of breast, prostate, and lung cancer to the bone. In the present paper, Ahr not only increased PTHrP expression, but was also involved in the L-Kyn-induced generation of PTHrP. These results are consistent with the findings that Ahr expression and constitutive activation (endogenous ligand-driven) in breast cancer cells correlate with tumor aggressiveness [32, 33] and control the expression of genes associated with tumor invasion [34]. However, the regulation of PTHrP gene expression is complex. It has been confirmed that there is a crosstalk between Ahr and TGF-β signaling that can promote PTHrP expression [23, 35]. While TGF-β signaling is regulated by many factors. Thus, PTHrP expression is likely regulated by many factors, not just Ahr. For example, miR-30b can inhibit the TGF-β-induced physiological and biochemical processes via targeting

Snail [36], which suggests that miR-30b may regulate PTHrP expression. Therefore, the many factors that control gene expression may have caused the only modest decrease in PTHrP expression after inhibiting Ahr.

Taken together, we have found that L-Kyn can promote PTHrP expression. Moreover, Ahr is involved in the L-Kyn-mediated increase in PTHrP expression in 95D cells. These results deepen the understanding of the formation of bone metastatic lesions by the tumor microenvironment.

Authors' Contribution Zhiqing Duan participated in the study design, performed partly experiments, drafted the manuscript, and analyzed the data. Jiangong Lu performed partly experiments and analyzed the data.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Research Involving Human Participants and/or Animals This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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