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ORIGINAL ARTICLE

Expression of HERG in musculoskeletal tumors with different degrees of malignancy*

Lu Gan, Mo Li (Co-first author), Tongtao Yang (Co-first author), Jin Wu, Junjie Du, Zhuojing Luo (✉), Yong Zhou (✉)

Department of Orthopedics, Air Force Medical University, Xi'an 710032, China

Abstract

Objective The expression of HERG in common bone tumors is scarcely reported and there is a lack of dedicated studies. This study aimed to investigated the expression of HERG in several common musculoskeletal tumors.

Methods Immunohistochemical staining, RT-PCR, and Western blotting were used to observe HERG expression differences in various tissues and cell lines.

Results HERG was differentially expressed in different malignant tumors, both at a differential protein level and localization within tumors. HERG was not expressed in normal bone tissue. The HERG inhibitor E-4031 markedly inhibited the proliferation of osteosarcoma cell lines.

Conclusion HERG was highly expressed in malignant tumors. Blocking of HERG can effectively inhibit the proliferation of bone tumors.

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Human Ether-a-go-go Related Gene (HERG) potassium channels play an important role in repolarization in cardiac action potentials. However, recent evidence has suggested a role for HERG in the proliferation and progression of multiple types of cancers, which may make it an attractive target for cancer therapy [1]. It has been confirmed that the existence of encoded potassium ion channels within tumor cell membranes has the ability to control the communication current of the cells. This can result in cell membrane depolarization at a deeper level due to specific biophysical properties [2]. HERG channels were reportedly expressed in tumor cell lines of various origins [3–4]. Additionally, HERG expression has been described in an array of human primary tumors including endometrial cancer, acute myeloid leukemia, and lymphocytic leukemia [5–7]. As is well known, tumorigenesis is a complex process. However, there is insufficient information regarding the expression of HERG in common bone tumors. Hernandez *et al* found that a significant anti-proliferative effect was associated with the HERG potassium channel in rat osteosarcoma cells [8]. The BKCa potassium channel has the ability to inhibit osteosarcoma growth [9]. However, it is still unclear what the relationship of the HERG potassium channel is to the phenotype of bone tumors. Specifically, the questions that remain are if differences exist in HERG expression levels in bone tumors of various origins as well as malignant bone tumors. Using clinical bone tumor specimens with different degrees of malignancy, this study reports the expression levels of HERG and its correlation with bone tumor malignancy.

Materials and methods

Immunohistochemistry

Specimen collection

With the consent of the patients themselves and their families, 60 bone tumor patients (38 males and 22 females) were collated according to the requirements of the Medical Association. Specimens were collected in the operating room of our department from June 2007 to December 2009. Twenty-five and 35 patients were

✉ Correspondence to: Zhuojing Luo. Email: zjluo@fmmu.edu.cn
Yong Zhou. Email: gukezy@fmmu.edu.cn

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characterized as having malignant and benign tumors, respectively.

Immunohistochemical staining

After routine de-waxing, tumor slices were treated with 3% hydrogen peroxide for 10 min and the antigen was retrieved using the microwave method. The primary antibody (CHEMICON Inc., AB5908-200 UL) was added and incubated at 4 °C overnight. The goat anti-mouse secondary antibody was subsequently added. Samples were washed with PBS and stained with DAB and HE. Slices were observed using a microscope camera, which was connected to a computer with auxiliary programs to capture images.

HERG mRNA expression level detection within different tumors using RT-PCR

Samples of approximately 1 g were selected for total RNA extraction and treated with TRIZOL (Invitrogen, USA). Total RNA extraction was conducted with an extraction kit (BETEKE, China).

cDNA synthesis

The TOYOBO First Strand cDNA Synthesis Kit was used. Briefly, 20 μL of reaction mix, 1 μL of Oligo (dT) 20, 1 μL of template RNA, and 9 μL of DEPC water were centrifugally mixed. After 5-min incubation at 65 °C, samples were put on ice and 4 μL of 5 × buffer, 1 μL of RNA inhibitor, 2 μL of dNTP mixture, and 1 μL of Rever TraAce were added. After centrifugal mixing, samples were incubated at 25 °C for 5 min, and 1 μL of reverse transcriptase was added. The reaction then proceeded at 30 °C for 10 min, 42 °C for 20 min, 85 °C for 5 min, and 4 °C for 5 min.

Polymerase chain reaction

Using β-actin as a control, HERG was amplified with PCR. The primers were as follows: HERG, 5'-AGA TGC TGC GGG TGC GG-3', 5'-CGA AGG CAG CCC TTG GTG-3'; β-actin, 5'-TCC ACC TTC CAG CAG ATG TG-3', 5'-GCA TTT GCG GTG GAC GAT-3'. The reaction system volume was 25 μL. The PCR reaction conditions for HERG were 98 °C for 10 min, 72 °C for 1 min, 94 °C for 40 s, 66 °C for 40 s, and 72 °C for 17 s for 35 cycles, with a final extension at 72 °C for 10 min. The PCR reaction conditions for β-actin were 94 °C for 2 min, 94 °C for 30 s, and 68 °C for 30 s for 35 cycles, with a final extension at 68 °C for 7 min.

Identification and analysis of PCR products

PCR products (20 μL) were separated using electrophoresis with a 15 g/L agarose gel with 70 V voltage electrophoresis. Observations and photographic results were recorded. Bands of the target and control genes were analyzed by BIORAD UNIVERSAL HOOD II-type gel imaging pixel.

Protein level detection of HERG (Western blot)

Protein extraction

Tissue from surgical excisions was weighed and cut into several smaller pieces. The pieces were put into a homogenizer per net weight, and the appropriate volume of lysis buffer was added. Supernatants were collected after centrifugation. Laemmli buffer was added to the supernatants, which were then further homogenized. The mixed sample was centrifuged at 10,000 g for 10 minutes before the supernatant was transferred to another tube. The sample was then ready for electrophoresis. Protein (50 μg) was loaded per lane for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Samples were subsequently transferred to a nitrocellulose membrane. The membrane was blocked with skimmed milk powder and rinsed with TBST buffer 3 times for 10 min each time. Rabbit anti-HERG polyclonal antibody was added and incubated at 4 °C overnight. Horseradish peroxidase-labeled goat anti-rabbit secondary antibody was added and incubated for 2 h with oscillation at room temperature. A chemiluminescence color development system with light-sensitive X-ray film imaging was used to visualize the protein bands. The experiment was repeated twice.

Effect of K+ channel inhibitors on the proliferation of human osteosarcoma cells

Method

Cells in the logarithmic phase were collected and the concentration of the cell suspension was adjusted. 100 μL of the cell suspension containing 1,000–10,000 cells was added to each well. These were incubated in 5% CO_2 at 37 °C until the bottom of the wells were covered with single cell layer. A drug inhibitor was added after the adhesion of the cells. Cells were again incubated in 5% CO_2 at 37 °C for 16–48 h. The medium was carefully removed from each well and 50 μL of MTT solution (5 mg/mL or 0.5% MTT) was added into each well with a subsequent 4 h incubation. 150 μL of DMSO was added to each well. The plate was shaken with a low-speed oscillation for 10 min to fully dissolve the DMSO crystals. Absorption at OD 490 nm was measured with an enzyme-linked immunoassay instrument. The blank well and control well were used for calculations.

Results

In order to effectively observe the expression of HERG in tumor tissue, 57 cases of patients with bone tumors were selected in accordance with the common collate classification of bone tumors (Table 1). A normal human bone specimen was selected as a control. Immunohistochemical staining was used to detect the expression levels of HERG as well as western blot. RT-

Table 1 Classification of common bone tumors

Category	Benign (cases)	Malignant (cases)
Osteoplastic tumor	Benign osteblastoma (3)	Osteosarcoma (12); osteblastoma (1)
Cartilaginous tumors	Enchondrosis (1), chondroma (2), benign chondroblastoma (1), cartilage Fibroma (1)	Chondrosarcoma (6); malignant chondroblastoma (3); mesenchymal chondrosarcoma (2); dedifferentiated chondrosarcoma (0)
Multinucleated giant cell	Benign bone giant cell tumor (1)	Malignant bone giant cell tumor (2)
Marrow source tumor		Ewing sarcoma (2)
Connective tissue source tumor	Non-ossifying fibroma (1)	Malignant fibrosarcoma (2) squamous cell carcinoma (2)
Vascular oringal tumor	hemangioma	Hemangioendothelioma (0), hemangiopericytoma (0)
Adipose tissue oringin tumor	Lipoma (3)	Liposarcoma (0)
Nerve tissue oringin tumor malignant neurilemoma	Neurilemmoma (2)	Malignant neurilemoma (1) Chordoma (1)
Tumor-like lesion	Cyst (1) bone fibrous dysplasia (1) inflammatory granulation tissue (2)	

PCR was used to detect HERG mRNA expression levels with semi-quantitative software. The results showed that the expression of HERG was related to the degree of malignancy of bone tumors. HERG was not expressed in other samples except for a low degree of expression in the giant cell tumors of I-level, non-ossifying fibroma and chondroblastoma. A high expression of HERG with differing levels was seen in malignant tumors.

Immunohistochemical staining

Immunohistochemical staining showed that there was a significant difference between benign and malignant bone tumors for HERG expression levels. In order to measure this expression difference in positive tissue samples, software was used to quantify the positive stained cells (yellow earth). The grade was divided into 0, 1+, 2+, 3+ and 4. The results showed that 91.3% of benign lesions were negative, with the expression of two cases scoring just 1^+ (Fig. 1).

As seen in Fig. 1, HERG was not expressed by the majority of the benign lesions. Low HERG expression was observed in bone giant cell tumors of I-level, non-ossifying fibroma and osteoblastoma origin. A relationship was shown between the HERG expression results and the origin of tumors and their malignancy. That there was high HERG expression levels in all malignant tumors showed that there was a relationship between benign and malignant tumors and their genesis (Fig. 2). This could be explained, as Fig. 1 demonstrates, by the lower level of HERG expression in some benign lesions as well as normal tissue samples. HERG participates in the regulation of cell proliferation and differentiation and so it is reasonable that there is a lower level of HERG expression in mesenchymal tissue excitatory cells, as is observed in Fig. 1d, 1e and 1h, as well as benign lesions.

mRNA expression levels by RT-PCR

It was shown in the same patient samples that HERG mRNA expression levels corresponded with the immunohistochemical results (Fig. 3). In group A (benign tumor samples), expression of HERG was not detected, whereas a faint expression was observed for other potassium channels. It is hard to explain the relationship of HERG with the cell cycle. One suggested hypothesis was that the regulation of the cell volume plays a key role in the concentration of intracellular fluid, which is important for the cell's primary metabolism. Our results provide evidence that HERG is expressed at different levels in different malignant bone tumor cells. There is a stronger expression of HERG in malignant tumor cells. A number of other Kv family genes (for example Kv1.3) were not overexpressed. Further investigation is needed to understand the reason for this difference and its relationship with the cell cycle.

Western blot

The results of the western blot clearly showed the expression of HERG in both groups (Fig. 4).

MTT assay with HERG inhibitor to assess the proliferation of osteosarcoma cell lines

E-4031 (a HERG channel inhibitor) and different concentrations of non-specific inhibitor 4-AP (a Kv inhibitor) were added to three human osteosarcoma cell lines. After 48 hours incubation, an MTT assay was performed. Results showed that the inhibition of tumor cell proliferation occurred after incubation with the HERG channel inhibitor E-4031 (Fig. 5), although no

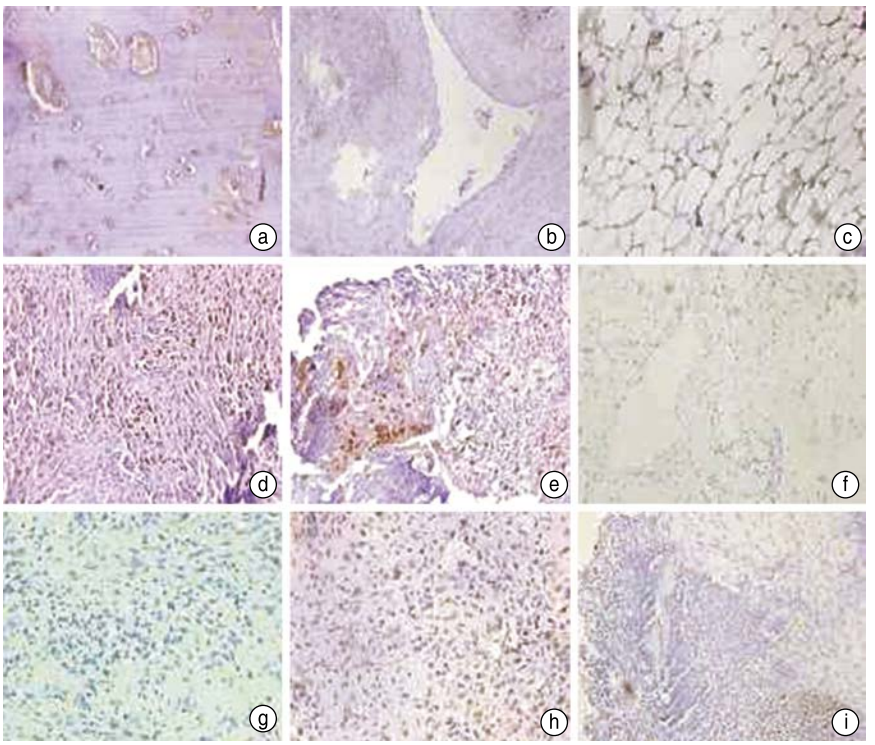

Fig. 1 Immunohistochemical staining for HERG in benign bone tumors. Detection of HERG in the 23 selected cases of benign tumors: normal bone tissue (a) –0; bone cyst (b) –0; fatty tumor (c) –0; bone giant cell grade I tumors (d) –1+; non-ossifying fibroma (e) –1+; hemangioma (f) –0; nerve sheath tumors (g) –0; osteoblastoma (h) –1+; inflammatory granulation tissue (i) –0

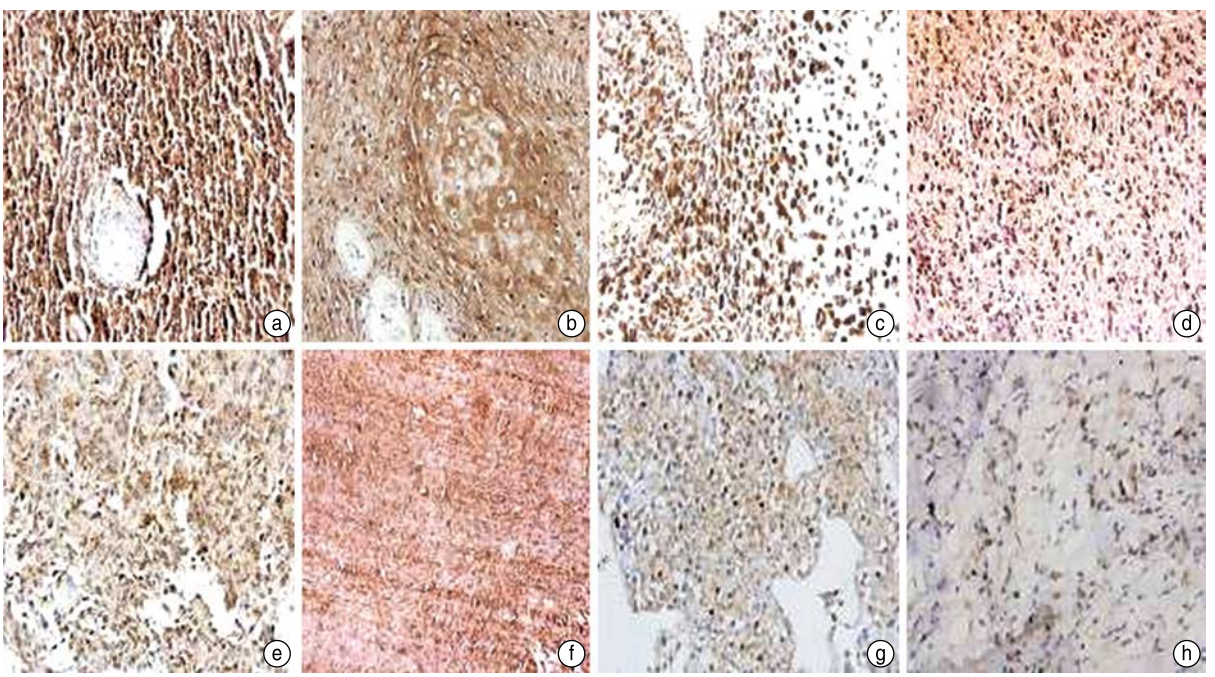

Fig. 2 Immunohistochemical staining for HERG in malignant bone tumors. Detection of HERG in the 34 selected cases of malignant tumors: Ewing sarcoma (a) –4; squamous cell carcinoma (b) –4; osteosarcoma (c) –4; malignant fibrous tissue sarcoma (d) –4; giant cell tumor II level (e) –3+; malignant peripheral nerve sheath tumor (f) –4; alveolar soft tissue sarcoma (g) –3+; sacral chordoma (h) –2+

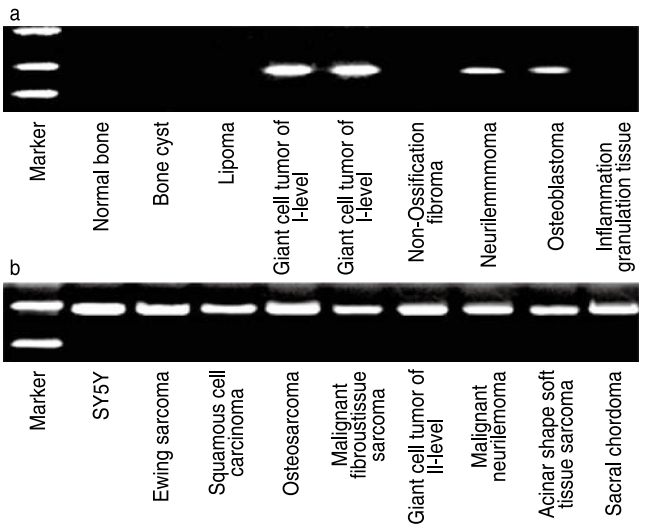

Fig. 3 The expression differences of HERG in common orthopedic tumors. Samples were divided into a benign group and a malignant group. Normal human bone tissue was used as a negative control (a, lane 1) and human neuron tumor cell line SY5Y was used as a positive control (b, lane 1)

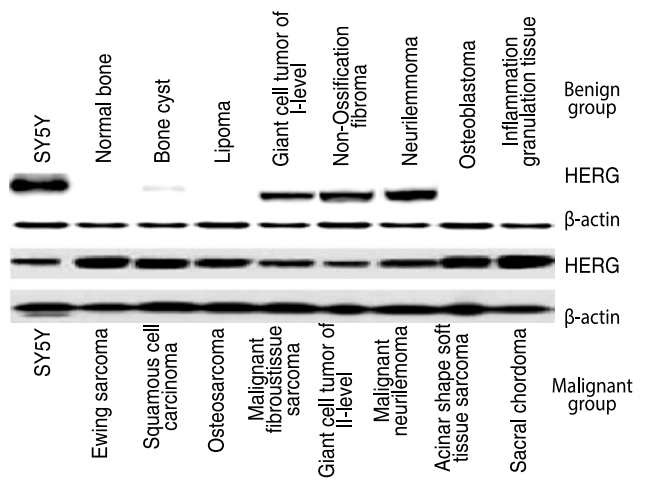

Fig. 4 HERG different expression levels in different bone tumors by western blot analysis. Specimens were divided into a benign and a malignant group. Normal human bone tissue was used as a negative control (a, lane 1) and human neuroblastoma cell line SY5Y was used as a positive control (b, lane 1)

statistical significance was found ($P > 0.05$). However, a significant difference was found with the non-specific Kv channel inhibitor 4-AP on cell proliferation at concentrations 3 mmol and 5 mmol (Fig. 6) ($P < 0.01$).

Discussion

Originally detected in fibroblast cell tumors, HERG is responsible for controlling cell membrane resting potential [10–13]. Potassium ion channels can regulate the proliferation of arterial pulsation and vascular smooth

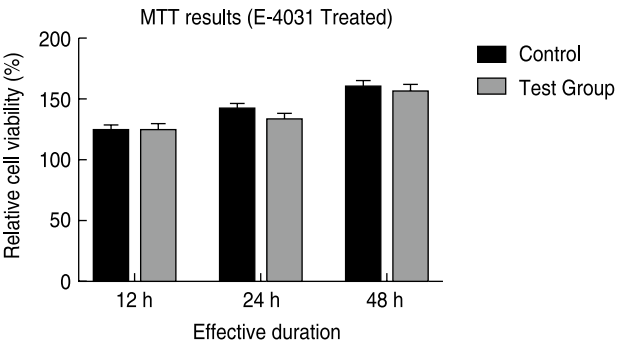

Fig. 5 Inhibition of three osteosarcoma cell lines with HERG inhibitor E-4031

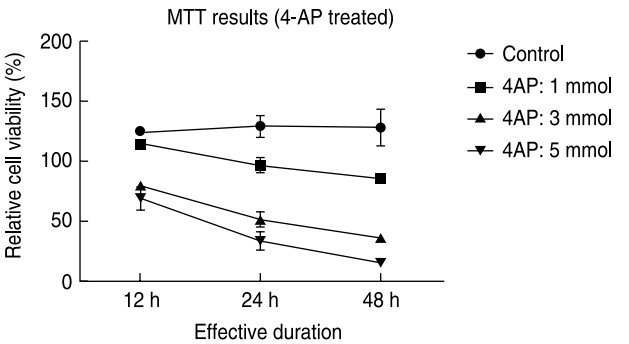

Fig. 6 Inhibition of three osteosarcoma cell lines with different concentrations of 4-AP

muscle cells [14]. Potassium ion channels have also been associated with playing an important role in gastric cancer [15]. A variety of the potassium ion channels are expressed in melanoma tumors and potassium ion channel inhibitors have the ability to inhibit the proliferation of melanoma cells [15] as well breast cancer cells [16]. Further, it was reported that blockers of potassium ion channels could effectively inhibit the proliferation of human colon cancer cell lines including SW1116, LoVo, Colo320DM, and LS174t [17]. Our study results are compatible with such previous reports. It has been well established that potassium ion channels are differentially expressed *in vitro* within multiple tissue types. However, the mechanism underpinning the involvement of potassium ion channels in the occurrence and development of tumor cells and their proliferation and differentiation remains unclear. To date, the expression of HERG in common orthopedic tumors has not been reported. In this study, which involved all common bone tumor types, 60 clinical cases were useful in observing HERG expression in bone tumors. These specimens also exposed differences between HERG expression in malignant tumors and normal bone tissue. The results showed that there is a distinct HERG expression pattern in benign

lesions and malignant orthopedic lesions. The hypothesis was consistent with the experimental results.

Using RT-PCR and western blot, the expression of the HERG gene was detected at both RNA and protein levels. As in previous clinical studies, an association between HERG expression, a potassium ion channel with electrophysiological properties, and tumor proliferation was found in osteosarcoma samples. Sample classification was differentiated according to previous research. Significant statistical differences were shown in HERG expression in different origin tissues of the tumors.

Based on the results above, it can be concluded that: (1) potassium ion channels play an important role in the progression of the cell cycle in cancer cells; (2) potassium ion channels that are involved in the progression of the cell cycle of different tumors belonging to different subtypes. For example, Kv channels are more important in oligodendrocyte progenitor cells, KCa channels may be more important in breast cancer cells, and KATP channels may be critical for human bladder tumor cancer cells [33].

HERG has attracted attention from those interested in the study of potassium ion channels. HERG may play a role in the diagnosis of bone cancer and an understanding of its mechanism of action and subsequent blockade could prevent the development of tumor cells with the potential to become a novel treatment and guide new directions of research. Further study is required into the mechanism of HERG and its applications.

Techniques such as patch-clamp, RT-PCR, and immunohistochemistry have highlighted the prevalence of Kv1.1 channels in human breast cancer cell line MCF-7 by Ouadid-Ahidouch *et al* [18]. Currently, potassium ion channels can be completely blocked by TEA, a non-specific inhibitor of Kv channels. Cell proliferation can be inhibited by Kv1.1 potassium channel blocker α-specific toxins. Preussat *et al* [19] found that Kv1.3 and Kv1.5 channels were differentially expressed in a variety of different types of glioma tissues. Sukuzi *et al* [20] found that Kv2.1 channels and HERG are strongly expressed in some cervical cancer cell lines. To some extent, cervical cancer cell proliferation was inhibited by Hanatoxin-1, which inhibits the Kv2.1 channel, but had no effect on the proliferation of cervical cancer cells that did not express the Kv2.1 channel.

HERG is differentially expressed in different species such as mice, cows, and humans [21]. In humans, EAG and ERG channels are known as the Human ether-a-go-go gene (HEAG and HERG, respectively). Further study has shown that the HERG potassium channel has a special status in the family and a close relationship with tumor development. It is worth noting that HERG is expressed in the early stages of embryonic development. To study different HERG expression patterns in tumors, 60 patient samples were collected. Bone tumors (particularly the malignant bone tumor osteosarcoma specimens) showed altered proliferation when their potassium ion channels were blocked, as expected. Using the HERG channel inhibitor E-4031, tumor cell proliferation inhibition was confirmed with MTT, although the results were not statistically different (P>0.05). However, the non-specific Kv channel inhibitor 4-AP had a significant effect on cell proliferation at concentrations of 3 mmol and 5 mmol with statistically significant differences (P<0.01). This study reveals that HERG is highly expressed in common tumors such as malignant bone tumor osteosarcoma. Other research has studied the physiological properties and biological behavior of HERG [22, 23]. Newer technologies such as microRNA and RNAi have been used in these studies [24, 25]. Currently, work is ongoing to understand the mechanism underpinning HERG and other mediators interact with it. It is hoped that future pharmacological developments using HERG will provide a reliable target to control various types of cancer including osteosarcomas.

Conflicts of interest

The authors declare no potential conflicts of interest.

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