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TRESK potassium channel in human T lymphoblasts

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ABSTRACT

TRESK (*TWIK-related spinal cord K*$^+$) channel, encoded by KCNK18 gene, belongs to the double-pore domain K^+ channel family and in normal conditions is expressed predominantly in the central nervous system. In our previous patch-clamp study on Jurkat T lymphoblasts we have characterized highly selective K^+ channel with pharmacological profile identical to TRESK. In the present work, the presence of KCNK18 mRNA was confirmed in T lymphoblastic cell lines (Jurkat, JCaM, H9) but not in resting peripheral blood lymphocytes of healthy donors. Positive immunostaining for TRESK was demonstrated in lymphoblastic cell lines, in germinal centers of non-tumoral lymph nodes, and in clinical samples of T acute lymphoblastic leukemias/lymphomas. Besides detection in the plasma membrane, intracellular TRESK localization was also revealed. Possible involvement of TRESK channel in lymphocyte proliferation and tumorigenesis is discussed.

1. Introduction

Leukemias and lymphomas correspond to the large group of lymphoproliferative disorders. In leukemias, predominantly bone marrow is affected, and circulated tumor cells are present. In lymphomas, lymph nodes, spleen and other solid organs are involved, although lymphoma cells may also circulate. Lymphoblastic leukemias and lymphomas are neoplasms of precursor cells, or lymphoblasts. Based on morphologic, genetic and immunophenotypic features, World Health Organization classifies lymphoblastic lymphoma and acute lymphoblastic leukemia (ALL) as the same disease [1].

Jurkat cell line was established from a child with T cell acute lymphoblastic leukemia (ALL) [2]. This model system contributed significantly to determine the mechanisms of differential susceptibility of ALL to drugs and radiation [3]. Recently, applying patch-clamp technique to Jurkat cells we characterized the background current previously unknown in lymphocytes. It was generated by voltage-independent channels with a high K^+ selectivity. Pharmacologic characteristics of this current were very similar to TRESK (TWIK-related spinal cord K^+ channel, KCNK18) that belongs to the double-pore domain K^+ (K2P) channel family [4].

In normal physiological conditions, TRESK is predominantly expressed in dorsal root ganglion (DRG) neurons in rodents [5] and in spinal cord in humans [6,7]. Although TRESK mRNA was also detected in rodent thymus and spleen [7], there is no data on human lymphoid tissues.

TRESK channel provides hyperpolarizing background current [6]. In our experiments with Jurkat cells, during recording of the run-up of TRESK-like current, stepwise membrane hyperpolarization was observed [4]. Membrane hyperpolarization was shown in lymphocytes during cell cycle progression from G1 to S phase [8]. It seems to be an important factor in maintaining sustained Ca^{2+} influx in activated lymphocytes [9,10]. Therefore, described in our previous studies TRESK-like channel may provide hyperpolarizing background current and contribute significantly to the maintenance of proliferating status of leukemic cells. Remarkably, TRESK activation is mediated by the calcium/calmodulin-dependent serine/threonine phosphatase, calcineurin and inhibited by calcineurin inhibitors, such as cyclosporine A and FK506 [11]. Both compounds are widely used immunosuppressant drugs, and the mechanism of suppression was first discovered in Jurkat cells [12]. A constitutively high level of calcineurin activity was demonstrated in leukemic cells [13]. Intracellular loop of TRESK was shown to possess highly conserved amino acid sequence similar to the docking site for calcineurin of nuclear factor of activated T-cells (NFAT), and calcineurin is recruited to this motif during TRESK activation [14].

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Based on our findings [4,15] and on unique characteristics of TRESK among K2P channels family [14], Han and Kang proposed TRESK as a potential target to treat T-cell mediated immune dysfunctions [16]. Yet, for clinical studies, the molecular identity of TRESK- like channel in Jurkat cells needs to be confirmed. It is also of special importance to reveal immune disorders, in which TRESK channel is involved. This would justify further pharmacological studies, targeting TRESK channels, for T-cell mediated immune disorders treatment. Present study was designed to address these specific questions.

2. Materials and methods

2.1. Cells

Jurkat (clone E6-1), J.CaM 1.6, and H9 lines were obtained from American Type Culture Collection (Manassas, VA, USA) and grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1 mM L-glutamine, 1 mM sodium pyruvate (all reagents from Invitrogen) at 37 °C in a humidified atmosphere with 5% CO_2. Cells growing in log phase at a density of 1.0–1.5 $\times$ 10^6/ml were used for experiments.

Mononuclear cells were harvested from the peripheral blood samples of healthy volunteers by Histopaque gradient centrifugation (Sigma–Aldrich 10771). The protocol was approved by the local Bioethical Committee.

2.2. RNA extraction and real-time RT-PCR

Total RNA from cultured cells (1 $\times$ 10^6 cells/sample) and human spinal cord tissue was purified using High pure RNA tissue kit (Roche 12033674001, Germany), treated with DNase (Invitrogen, San Diego, CA), **r**etro-transcribed and amplified by real-time PCR using the One-Step SuperScript® qRT-PCR Universal Kit (Invitrogen). A RNA sample from each assay was processed equally, but without reverse transcriptase (replaced with water), to obtain the DNA background control (DBC). Autopsy samples of human spinal cord were used as positive control. Real-time RT-PCR was performed on an Eco Real-Time PCR System (Illumina, Inc., San Diego, CA, USA) with oligonucleotides and probe designed for TRESK cDNA, using the ProbeFinder Assay Design Software (Universal Probe Library: Roche Diagnostics, Mannheim, Germany). The sequences of the primers were: forward, 5′-atactccatcatcagcaacctg-3′; reverse, 5′-agggggatgtccaacctct-3′; and Roche universal probe for human No. 56 (FAM). The program used for reverse transcription was as follows: 50 °C for 15 min and 2 min at 92 °C. Amplification conditions were: 95 °C, 56 °C and 60 °C for 15 s, 15 s and 30 s, respectively, for 45 cycles. A standard curve with a known logarithmic number of DNA copies from 0 to 10^6 copies was used for quantification. Numbers of transcript copies were expressed per nanogram of total RNA. Tubes with DBC were used to normalize the data of each sample to zero.

2.3. Antibodies

During the initial period of our study primary polyclonal antibodies produced in goat and raised against three different epitopes of hTRESK protein were purchased from Santa Cruz Biotechnology, Inc: sc-51235 (aa 52–102), sc-51238 and sc-51239 (aa 200–250). Dilution for Western blot was 1:200, and for immunofluorescence and immunohistochemistry 1:100, in line with recommendation of the manufacturer. Later, we used also anti-TRESK antibodies purchased from other manufacturers: polyclonal antibodies against center intracellular region of TRESK protein produced in rabbit (Abcam ab10641, ProSci 5557). According to the manufacturer recommendation, dilution for Western blot was 1:500 and for immunohistochemistry 1:300. Anti-Kv1.3 polyclonal rabbit antibodies were from Alomone labs (APC-101) and used in dilution 1:300 both for Western blotting and immunohistochemistry. Rabbit polyclonal antibodies to GAPDH from Abcam (ab9485) as constitutively expressed proteins were used in Western blot at dilution of 1:2000. Secondary antibodies directed against primary antibodies produced in goat were: rabbit anti-goat IgG antibodies conjugated with horseradish peroxidase (HRP, 1:3000, Sigma A8919), and rabbit anti-goat IgG antibodies labeled with AlexaFluor 568 (1:1000, Molecular probes A11079, Ex/Em: 578/603). Secondary antibodies directed against primary antibodies produced in rabbit were: goat polyclonal antibodies to rabbit IgG- H&L coupled with HRP (Abcam, ab6721).

2.4. Protein isolation and Western blot

To obtain whole cell extract, Jurkat cells were lysed with NP-40 buffer containing 150 mM NaCl, 1% NP-40, 50 mM Tris (all from SIGMA), protease inhibitor cocktail (Thermo Scientific 78412), pH 8.0. Membrane proteins were extracted using Mem-PER® Eukaryotic Membrane Protein Extraction Reagent Kit (Pierce, 89826). Protein samples were purified using PAGEprep™ Advance Kit (Pierce, 89888). Protein concentration in the samples was determined by the BCA method (Sigma, product code BCA1 and B9643). Protein fractions (100 μg/sample) were separated on 10% SDS–polyacrylamide gels, and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were treated by SuperSignal Western Blot Enhancer (Pierce, 46640) and blocked with SuperBlock T20 (PBS) Blocking Buffer (Pierce, 37516). Then membranes were cut in strips, and every strip was incubated with corresponded primary antibody overnight at 4 °C with agitation. Next day, after washing strips with TTBS, they were treated with corresponding secondary HRP-conjugated antibodies. Antigen–antibody complexes were visualized by Metal Enhanced 3,3'-diaminobenzidine (DAB) Substrate Kit (Thermo Scientific 34065). Protein band mobility was determined using pre-stained broad range protein standard (Bio-Rad catalogue No 161-0318) and the Gel Pro Analyzer system (Media Cybernetics, MA, USA).

2.5. Immunofluorescence imaging

Cells were immobilized on poly-L-lysine pre-coated coverslips and fixed with freshly prepared 4% paraformaldehyde in PBS, pH 7.4 for 10 min at 25 °C. After several washes with PBS, cells were permeabilized with 0.1% PBS- Tween-20 for 20 min and treated with Image-iT FX Signal Enhancer (Invitrogen 136933). Nonspecific binding was blocked by treatment for 1 h in the following buffer: 1% BSA, 0.3 M glycine, 10% normal serum of the host species of secondary antibody. Samples were then incubated overnight with corresponding primary antibodies, followed by incubation with secondary antibodies. Coverslips were mounted on a slide with ProLong Gold antifade solution contained DAPI (Invitrogen 36935). The images were obtained by confocal microscopy using an LSM 700 system (Carl Zeiss) coupled to an inverted Observer.Z1 microscope, equipped with 40x and 63x oil-immersion objectives, 405 and 555 nm diode lasers (10 mW), and ZEN imaging software. The settings were always kept identical. Specifically: laser power 2%, pinhole Φ = 1.00 AU; detector gain = 685; line step: 1; scan speed: 6; data depth: 12 bit; mode: line; method: mean; scan number: 2; zoom: approx. 5 for single cell images. For negative controls, samples were treated as described above except that they were not exposed to primary antibody. No positive signal was revealed in these samples.

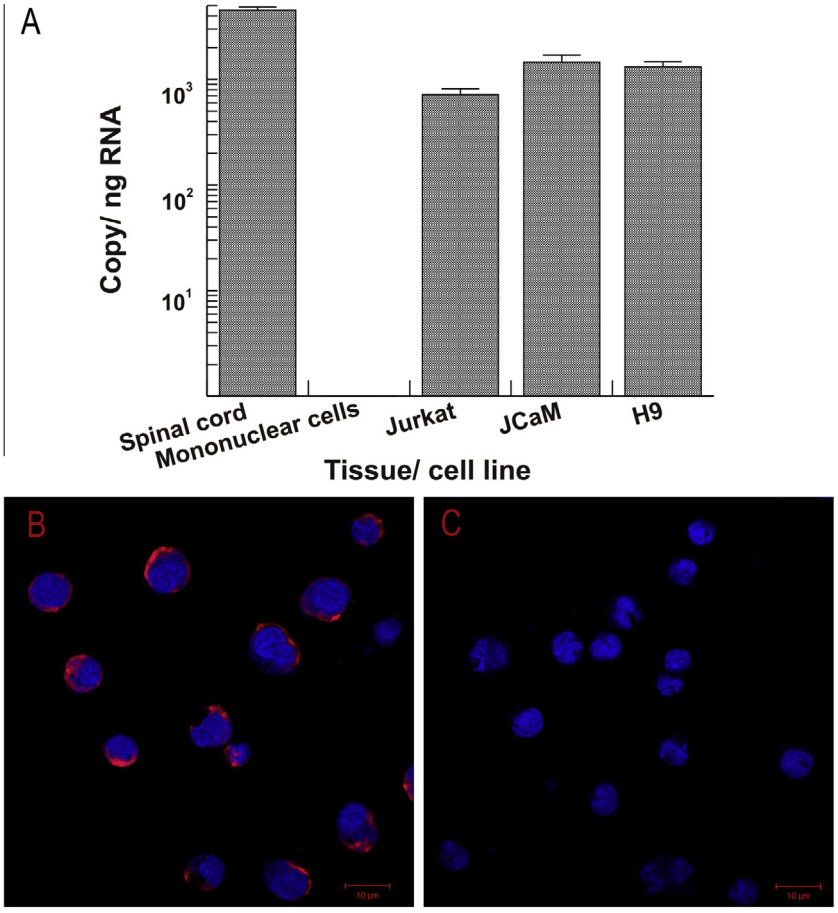

Fig. 1. KCNK18 mRNA and TRESK protein are present in lymphoblastic cell lines, but not in mononuclear cells of healthy donors. (A) Quantification of KCNK18 mRNA in human mononuclear cells and lymphoblastic cell lines. Spinal cord tissue was used as positive control. Error bars indicate the standard deviation of mean ($n = 3$). (B, C) Immunofluorescence images for TRESK (red): positive staining in Jurkat lymphoblasts (B) and negative staining in mononuclear cells of healthy donors (C). Nuclei: DAPI (blue); scale bar = 10 μm. (For interpretation of references to color in this figure legend, the reader is referred to the web version of this article.)

2.6. Tumor specimens and immunohistochemistry

Paraffin blocks not older than 5 years were obtained from the Departments of Pathology of the Centro Medico de Occidente (Instituto Mexicano del Seguro Social). Previous diagnostic was done based on expression of specific markers (TdT for lymphoblasts, CD3 for T lineage). Formalin-fixed, paraffin-embedded tissue was cut into 5 μm sections and mounted on silanized slides (Sigma–Aldrich 118K4369). Sections were deparaffinized, following xylene treatment and rehydration in descending alcohols. To block endogen peroxidase activity, the slides were incubated in 3% H_2O_2 in methanol for 15 min. Antigen retrieval was performed by heating the slides for 30 min in a steamer in 10 mM citrate buffer solution pH 6.0. Then the slides were cooled down to room temperature, and non-specific binding sites were blocked using 1% BSA (Sigma A1933) in TBS (30 min, room temperature). Slides were incubated with corresponding primary antibodies, rinsed with PBS (pH 7.6), incubated with secondary antibodies conjugated with HRP, and revealed with DBA. Then slides were counterstained with hematoxylin, dehydrated and mounted with balsam.

3. Results

3.1. TRESK is expressed in T lymphoblastic cell lines

Real time PCR technique was applied to confirm the presence of mRNA for hTRESK protein (encoded by KCNK18 gene) in lymphoid cell lines and mononuclear cells/lymphocytes isolated from blood of healthy donors. Since KCNK18 expression was confirmed previously in human spinal cord tissue [6,7], autopsy samples of human spinal cord were used as positive controls. Previously we have registered selective hTRESK-like potassium current in Jurkat T lymphoblasts [4]. Therefore, Jurkat cell line together with its derivate line J.CaM1.6 and not related to Jurkat H9 line (cutaneous $CD4^+$ lymphoma/Sezary syndrome) were used in the present work.

We found KCNK18 mRNA in samples of spinal cord and cell lines, but not in the mononuclear leukocytes of healthy donors (Fig. 1A). Similarly, presence of the TRESK protein was confirmed by immunofluorescence techniques in cell lines (Figs. 1B and 2B–D), but not in fractions of healthy monocytes/lymphocytes ($n = 10$, Fig. 1C).

Next we have undertaken experiments on subcellular localization of the TRESK protein. Two preparations, whole cell lysate and protein extract from cell membrane of Jurkat cells were used for Western blot experiments. We used commercially available antibodies from different providers that recognize distinct epitopes on the TRESK protein, yielding similar results. Usually, two bands of approx. 40 and 45 kDa (Fig. 2A) were detected in the whole cell lysate, similar to earlier observations [17]. Egenberger et al. [17] demonstrated that the band corresponding to higher molecular weight represents TRESK proteins modified post-translationally by N-glycosylation (predominantly mouse TRESK), phosphorylation, or O-glycosylation (hTRESK). These modifications seem to affect membrane targeting. In our experiments, the presence of the two bands corresponding to the TRESK protein was significantly

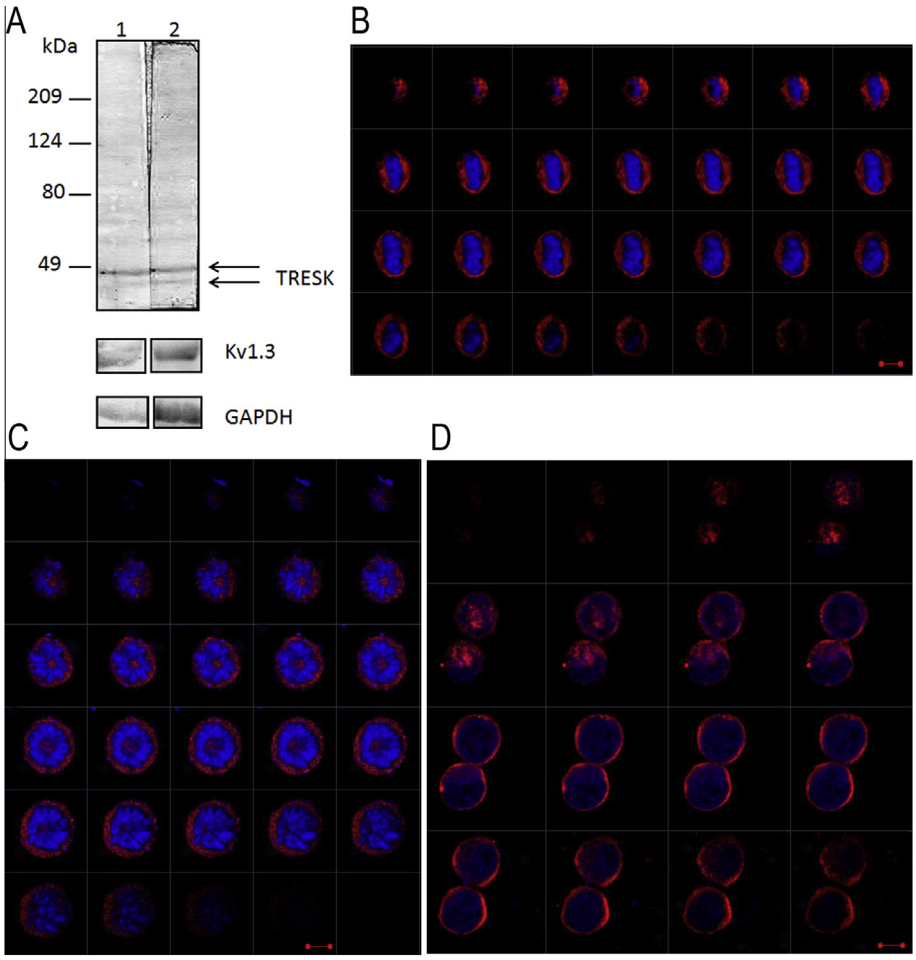

Fig. 2. Subcellular distribution of TRESK protein. (A) Western blot analysis with membrane protein preparation (lane 1) or whole cell lysate (lane 2) obtained from Jurkat cells. Primary antibodies for TRESK: sc-51238 (Santa Cruz Biotechnology, Inc). (B–D) High resolution confocal microscopy images of subcellular distribution of TRESK protein (red) in Jurkat (B), H9 (C) and JCaM (D) cells. The images are sequential 0.25 μm slices (B, C), and 0.45 μm slices (D). Primary antibodies for TRESK protein as in A. Secondary antibodies were labeled with AlexaFluor 568 (Molecular Probes A11079). Nuclei: DAPI. Bar: 5 μm. Note positive-stained cytoplasm present in invagination of cerebriform nuclei of H9 Sézary cell (C). (For interpretation of references to color in this figure legend, the reader is referred to the web version of this article.)

more pronounced in blots of the whole cell lysate, when compared with blots of membrane proteins. To obtain clearly visible bands, we were required to use at least 100 μg of protein extract for every sample. Voltage-dependent Kv1.3 K^+ channel is constitutively expressed in cell membrane of human lymphocytes [9]. In this work we detected Kv1.3 protein in both types of protein preparations. High resolution confocal imaging of the TRESK protein demonstrated its membrane and cytosolic localization, in line with Western blot data (Fig. 2B–D).

Overall, using different approaches we have demonstrated that Jurkat, JCaM, and H9 cells express TRESK. As H9 line is not related to Jurkat cells, we concluded that TRESK expression is not a clonal characteristic of Jurkat cells. Given the fact that mononuclear cells obtained from peripheral blood of healthy donors and contained high percentage of resting lymphocytes were negative for TRESK, we have hypothesized that TRESK expression may be related somehow with lymphocyte transformation and tumorigenesis. To test this hypothesis, following experiments were carried out.

3.2. TRESK expression in no-tumoral human tissues and T ALL/lymphomas

Immunohistochemical staining was used to visualize the TRESK protein in human tissues samples. As TRESK protein was previously reported in human central nervous system only, we first demonstrated the presence of TRESK protein in motor neurons from human spinal cord, with specific localization in plasma membrane, cytoplasm (Nissl substance) and perinuclear space (Fig. 3A–C).

To test whether TRESK is involved in tumorigenesis, samples from patients with T lymphoblastic LLA/lymphomas (TdT^+, $CD3^+$) were analyzed and compared with samples of non-tumoral lymphoid tissue. For this purpose, lymph node and tumoral samples were prepared on the same slide. Lymph nodes demonstrated moderate positive staining predominantly in germinal centers, where proliferation of antigen-activated lymphocytes takes place (Fig. 3D and E). In tumoral samples, positive staining with membrane and intracellular localization was present in majority of cells (Fig. 4A1, B1, C and D). Immunostaining for Kv1.3 channel broadly expressed in lymphocytes was also positive (Fig. 4A2 and B2).

4. Discussion

In the present work we have demonstrated the presence of KCNK18 mRNA in lymphoblastic T cell lines. TRESK protein was detected in germinal centers of non-tumoral human lymph nodes, in T lymphoblastic cell lines, and in clinical T ALL/lymphoma samples. TRESK was also detected functionally in Jurkat cells [4]. The fact that we did reveal neither TRESK mRNA nor protein in resting

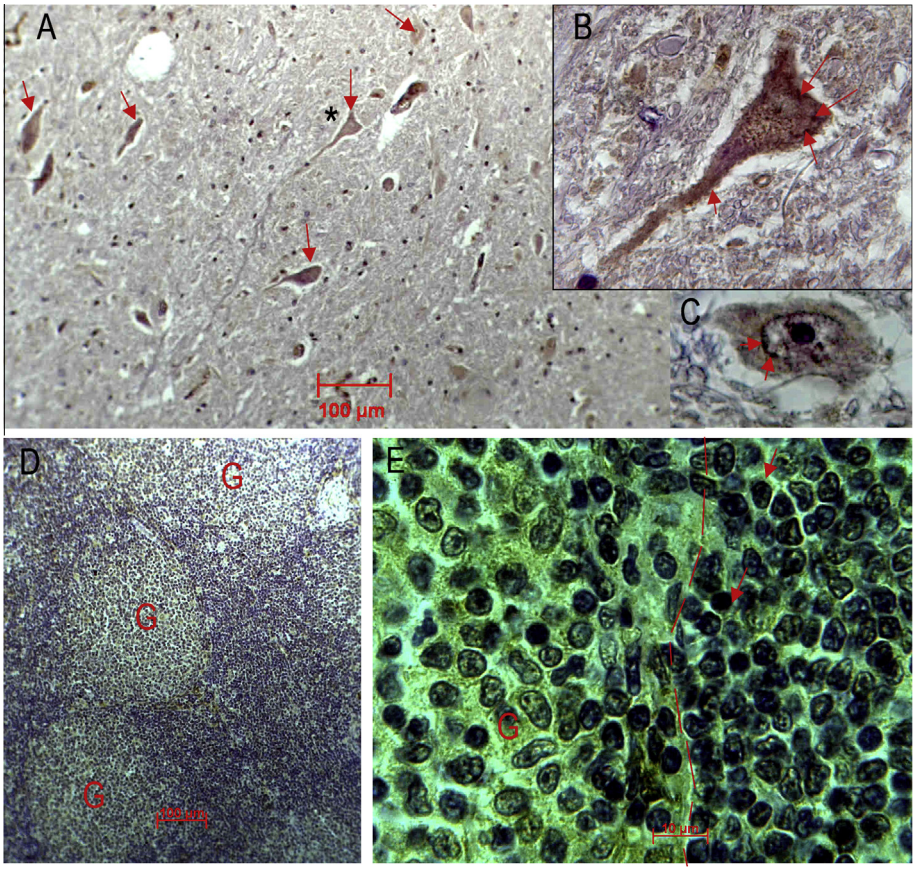

Fig. 3. Immunohistochemical staining of human spinal cord (A–C) and non-tumoral lymph node (D, E) tissue for TRESK protein. Primary antibodies: ProSci 5557. Secondary goat-anti-rabbit IgG antibodies were conjugated with HRP and revealed with DAB. Positively stained areas are brown. Cell nuclei were counterstained with Hematoxylin (blue). (A) Low magnification image of spinal cord. Positively stained motoneurons are indicated by arrows. Single neurons at high magnification are shown in (B) and (C). Neuron labeled with asterisk (∗) in A is shown in B and demonstrates positive staining in plasma membrane and cytoplasm (arrows). Positive staining in perinuclear space is indicated by arrows in (C). In non-tumoral lymph node at low (D) and high (E) magnification moderate positive staining of lymphocytes in germinal centers (G) is revealed. In (E), part of positively stained germinal center is at the left of dashed line. Some cells with positively stained cytoplasm are diffusely distributed in the areas out of germinal centers, in (E) indicated by arrows. (For interpretation of references to color in this figure legend, the reader is referred to the web version of this article.)

lymphocytes, obtained from peripheral blood of healthy volunteers, led us to suggest that TRESK is related to proliferation and neoplastic processes in immune system. In line with this hypothesis, expression of TRESK protein in activated $CD4^+$ lymphocytes was recently reported [18].

TRESK channel is highly selective for K^+ [6]. In lymphocytes, K^+ efflux generates driving force for Ca^{2+} influx, mandatory during proliferation [9]. The cells of leukemias/lymphomas proliferate independently of antigen receptor stimulation, and maintenance of sustained high Ca^{2+} level is critical in this case.

Leukemic cells may express K^+ channels, which are not characteristic for healthy leukocytes and are normally expressed in distinct tissues [19]. In adult mammals, physiological expression of *ether-à-go-go* (EAG, Kv10.1) channel is restricted to the brain, but aberrant expression is frequently observed in many solid tumors [20–22]. EAG is up-regulated in myelodysplastic syndromes (MDS), acute and chronic myeloid leukemia (AML, CML), where its expression strongly correlated with higher relapse rates and a significantly shorter overall survival [23]. In lymphatic leukemias, a related channel HERG (Kv11.1), which is normally expressed in heart [24], could be identified both in acute and chronic lymphoblastic (CLL) forms [25]. HERG was also found in non-lymphoid tumors of different histogenesis [26]. In ALL, HERG expression is highly correlated with resistance to chemotherapy [27].

Reports about role of members of K2P family in tumorigenesis were published earlier, including oncogenic potential of TASK3 in several types of carcinomas [28] and TREK1 in prostate cancer [29]. Unique property of TRESK channel, not shared with other K2P family members, is its up-regulation by calcineurin [11]. Since calcineurin is an important participant of NFAT-dependent signal transduction during proliferation of normal and neoplastic lymphocytes [30], it may serve as a feedback link between maintenance of high Ca^{2+} level and NFAT activation.

So far biophysical and pharmacological characterization of the TRESK channel was performed mainly in heterologous models, using L929 cells [6], COS-7 cells [7], *Xenopus laevis* oocytes [11]. However, heterologous models may hardly provide information about physiological role of the channel. Up to the moment, native TRESK currents were recorded only in rat DRG neurons [5]. We have provided the first evidence about functional TRESK expression in lymphocytes [4,15].

In our earlier work TRESK currents were recorded in plasma membrane of Jurkat cells [4] using patch-clamp. Now, using immunodetection methods we have revealed location of the TRESK protein. The protein was localized in the plasma membrane as well as in cytoplasm and perinuclear space, both in cell lines and in clinical samples. Similar to our findings, the intracellular localization of EAG1 (Kv10.1) channel was also reported in tumoral and artificially transfected cells, notwithstanding the robust EAG1 current recorded in electrophysiological experiments [22,31]. It was argued that in heterologous systems and tumor cells the vast majority of Kv10.1 protein remains in intracellular pools, including

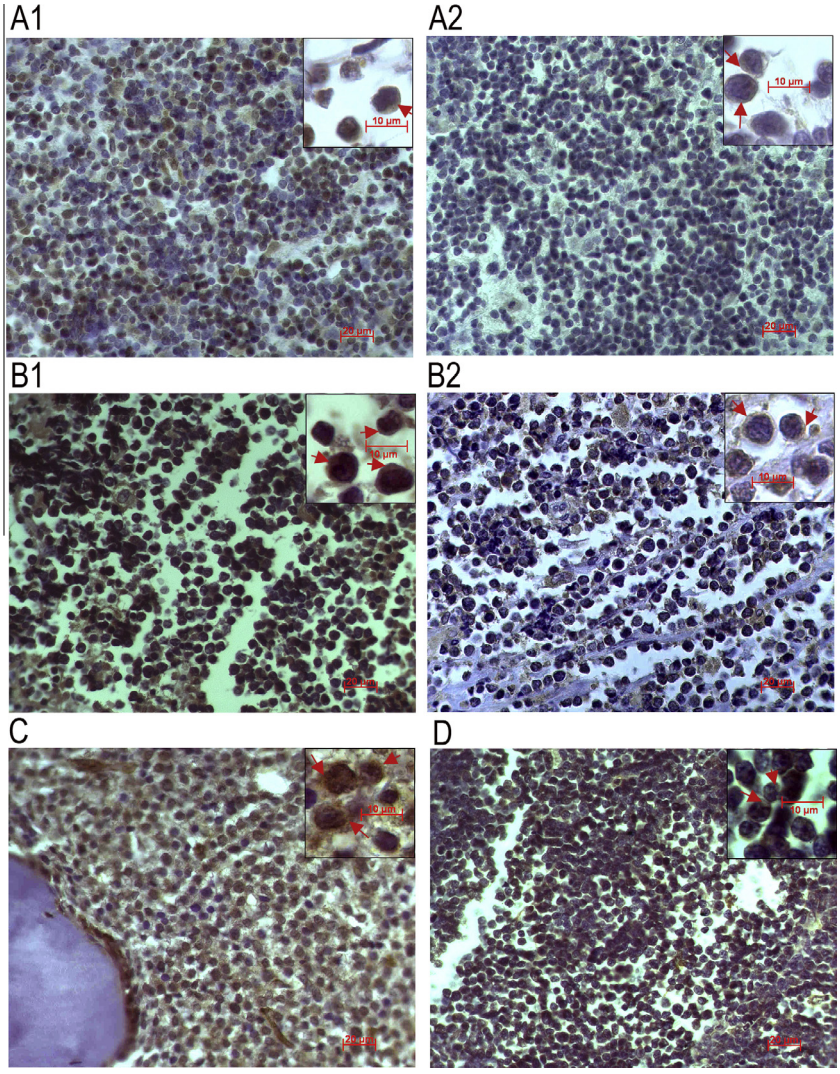

Fig. 4. Immunohistochemistry shows TRESK (A1, B1, C, D) and Kv1.3 (A2 and B2) expression (brown staining) in clinical samples. High magnification images of positively stained cells are present in inserts (indicated by arrows). A, B, D: pediatric acute T lymphoblastic lymphomas; C: acute T lymphoblastic leukemia, bone marrow biopsy. A1 and A2, B1 and B2, respectively, are prepared from the same paraffin block. (For interpretation of references to color in this figure legend, the reader is referred to the web version of this article.)

perinuclear region that was suggested to be an artifact of massive overexpression. However, Chen and colleagues [31] challenged the latter and demonstrated functional presence of Kv10.1 channel in the inner nuclear membrane (IMN). Moreover, inhibition of *de novo* protein synthesis by cycloheximide did not abolish the perinuclear localization, also arguing against an overexpression artifact. Functional K^+ channel present in IMN might contribute in the homeostasis of nuclear K^+. Interaction of Kv10.1 with heterochromatin was demonstrated. Regulation of concentration of nuclear K^+ as well as interaction with heterochromatin may affect gene expression and have a role in tumorigenesis.

In transformed lymphocytes, abnormally expressed K^+ channels may be involved in regulation of proliferation, differentiation, apoptosis, tissue invasion/metastasis and drug resistance [19]. Among K^+ channels considered as potential therapeutic targets in leukemias are Kv11.1 in AML and ALL, EAG1 channel in AML, CML, MDS, and KCa3.1 in B-lymphomas [32]. TRESK channel was also proposed as a target for treatment of lymphoid disorders [16]. In this regard our findings that T cell lymphoblastic leukemias/lymphomas express TRESK protein highlight its importance in the disease development and call for future studies.

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