A new human gene *KCNRG* encoding potassium channel regulating protein is a cancer suppressor gene candidate located in 13q14.3

D.V. Ivanov[a,b], T.V. Tyazhelova[a], L. Lemonnier[c], N. Kononenko[c], A.A. Pestova[a], E.A. Nikitin[d], N. Prevarskaya[c], R. Skryma[c], Y.V. Panchin[c,e,f], N.K. Yankovsky[a], A.V. Baranova[a,b,*]

[a] *Vavilov Institute of General Genetics, 3 Gubkina Str., 119991 Moscow, Russia*
[b] *Center for Biomedical Genomic and Informatics, Biology Department, George Mason University, 10910 University Blvd., MSN 4E3, Manassas, VA 20110 USA*
[c] *Laboratoire de Physiologie Cellulaire, INSERM EMI 0228, Bâtiment SN3, USTL, 59655 Villeneuve d'Ascq, France*
[d] *Hematology Research Center of Russia, Novozykovski pr.4a, 125167 Moscow, Russia*
[e] *Institute for Information Transmission Problems, 19 Bolshoi Karetny, 101447 Moscow GSP-4, Russia*
[f] *A. N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, 119899 Moscow, Russia*

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Abstract We report the primary characterization of a new gene *KCNRG* mapped at chromosome band 13q14.3. This gene includes three exons and has two alternatively spliced isoforms that are expressed in normal tissues and in some tumor cell lines. Protein KCNRG has high homology to tetramerization domain of voltage-gated K^+ channels. Using the patch-clamp technique we determined that KCNRG suppresses K^+ channel activity in human prostate cell line LNCaP. It is known that selective blockers of K^+ channels suppress lymphocyte and LNCaP cell line proliferation. We suggest that *KCNRG* is a candidate for a B-cell chronic lymphocytic leukemia and prostate cancer tumor suppressor gene.
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Key words: Tumor suppressor gene; B-cell chronic lymphocytic leukemia; Prostate cancer; 13q14.3; K^+ channel regulating protein

1. Introduction

Deletions in chromosomal band 13q14.3 have been previously associated with several different tumor types, including B-cell chronic lymphocytic leukemia (BCLL) [1] and prostate cancer [2], indicating importance of this region for suppression of a tumor development. The well-known tumor suppressor gene *RB1* located at chromosome 13q has been excluded as potential candidate for these diseases [3], implying the existence of one or more tumor-suppressor genes in this region.

Previously it was demonstrated that the region bordered by STS markers D13S1168 and D13S25 is frequently deleted in tumor cells form patients with BCLL [4,5]. The indicated area is immersed in a wider region that is deleted in prostate cancer samples [6,7]. Four candidate genes belonging to this region, namely *DLEU1* and *DLEU2* [4], *RFP2* [8], *C13ORF1* [9] were cloned. All candidates have been extensively checked for mutations by SSCP analysis and have failed to detect small mutations in any of these genes ([4] and our unpublished data on *C13ORF1*, and *RFP2*). These data suggest the existence of a different tumor suppressor gene, located in the area of interest.

In several cell types, enhanced K^+ channel gene expression or increased K^+ channel activity has been found to be associated with cell division [10]. Important role of voltage-gated K^+ channels in mitogenic growth control has been shown for human lymphocytes [11]. A high expression of EAG channels inhibited by physiological $[Ca^{2+}]i$ has been demonstrated in several human melanoma [12] and somatic cancer cell lines [13]. In human prostate cancer, LNCaP cells, the K^+ channels controlling proliferation are also inhibited by $[Ca^{2+}]i$ [14]. It has been suggested that TEA [15–19] and verapamil [20–23] inhibit the cancer cell proliferation by suppressing K^+ channel activity. We have previously demonstrated that in the androgen-sensitive prostate cancer cell line LNCaP the rate of the mitoses is decreased when voltage-activated K^+ channels are blocked implying that K^+ channels are involved in prostate cell proliferation [24,25].

Here we report genomic organization and tissue distribution of the new human gene *KCNRG* located between D13S1168 and D13S25. *KCNRG* encodes a soluble protein that shows strong homology to the cytoplasmic tetramerization domain (T1) of voltage-gated K^+ channels. We suggest that such protein might interfere with the normal assembly of the K^+ channel protein by binding to their tetramerization domain causing the suppression of the K^+ channels. We also examine the suppressor effect of *KCNRG* expression on the whole-cell potassium currents in human prostate cell line LNCaP by the patch-clamp method. The results of our research suggest that *KCNRG* is a strong candidate for a long-searched BCLL and prostate cancer tumor suppressor gene in human 13q14.3.

2. Materials and methods

2.1. Molecular cloning of a new gene KCNRG

To isolate a new gene *KCNRG* we have performed a BLASTN search of the expressed sequence tag (EST) database of GenBank with genomic sequence accession number AF440619 as a bait. We

*Corresponding author. Fax: (1)-703-993 4293.
E-mail address: abaranov@gmu.edu (A.V. Baranova).

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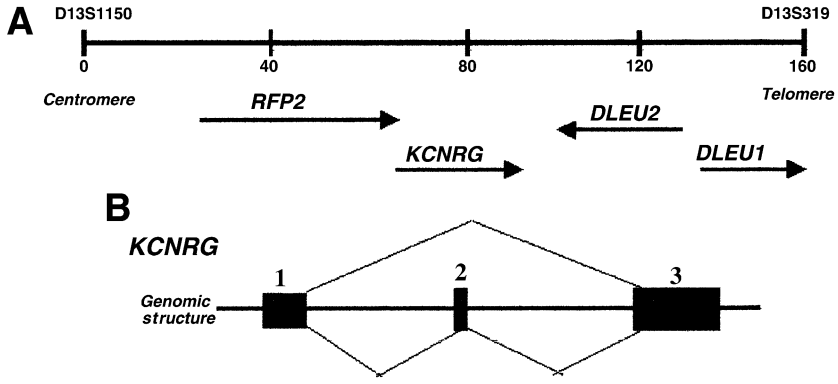

Fig. 1. A: Position of genes in the BCLL critical region in 13q14.3; B: genomic structure of *KCNRG*.

retrieved two expressed human sequences with the accession numbers BI770863 and BG537776. Corresponding cloned cDNAs IMAGE: 5207702 and IMAGE: 4690662 were obtained from ATCC USA. The plasmid cDNAs were isolated and completely sequenced using the following primers: NGf: 5′-GGTAGCCTCTAGTTTGAAGTG-3′; NGr: 5′-GAGGCAGTGGAAGTAAGGTC-3′; NGf871: 5′-GAAATGAAGTTGTCTATCCTC-3′; NGr871: 5′-GAGGATAGA-CAACTTCATTTC-3′; NGr110: 5′-GGAAAGATGTATTGAAGG-TAG-3′ and standard M13-rev and M13-forv sequencing primers.

2.2. RT-PCR analysis

A number of human poly(A) RNAs samples (Clontech, Palo Alto, CA, USA) were reverse transcribed to generate first-strand cDNAs with Advantage RT-for-PCR kit (Clontech, USA). PCR was carried out with these first-strand cDNAs, corresponding to 5 ng poly(A) RNA, with GenePakTM PCR Universal 100 (IsoGene, Russia) using the following primer pair NGf and NGr110. PCR was performed for 35 and 39 cycles at 94°C for 1 min, 58°C for 1 min and 72°C for 1 min. The amplified fragments were sequenced on both strands. As a control, GAPDH was amplified using specific primers (Clontech, USA).

2.3. Plasmid construction

KCNRG cDNA encompassing the entire coding region was synthesized by PCR amplification of cDNA insert from clone IMAGE: 4690662 using two gene-specific primers. A sense primer, containing a *Xho*I site at its 5′-end (NgreF: 5′-TATCTCGAGATGAGTAGT-CAGGAACTGGT-3′) and an antisense primer, containing a *Bam*HI site at its 5′-end (NgreR: 5′-TATGGATCCATACCTGTAATCC-CAGCTA-3′) were used. 500 ng of the pEGFP-N1 vector (Clontech, USA) and PCR product were digested by *Xho*I and *Bam*HI. The digested vector and the PCR products were gel purified, ligated together and cloned. The cDNA insert was sequenced to verify its identity and absence of mutation.

2.4. Electrophysiological studies

LNCaP cells from the ATCC were grown and prepared for electrophysiological experiments as described previously [14,25]. The whole-cell modes of the patch-clamp technique were used. This technique has been described in detail in previous publications [14,25].

The extracellular solution contained (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 0.3 Na_2HPO_4, 0.4 KH_2PO_4, 4 $NaHCO_3$, 5 glucose and 10 HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethano-sulfonic acid). The osmolarity of the external salt solution was adjusted to 310–315 mosmol l^{-1} with sucrose, and the pH adjusted to 7.3 ± 0.01 using NaOH. The internal solution contained (in mM): 140 KGlu, 1 $MgCl_2$, 0.5 $CaCl_2$, 8 EGTA (ethylene glycol bis β-aminoethyl ether-*N*,*N*,*N*′,*N*′-tetraacetic acid), and 5 HEPES (pH 7.2 ± 0.01 with KOH), osmolarity 300 mosmol l^{-1}. We have previously shown that the K^+ channel open probability decreased as internal free Ca^{2+} was augmented from 0.01 μM in the standard internal solution to 0.2–1 μM [14]. Therefore, in all the experiments in this study, the free Ca^{2+} concentration for the solutions applied from the inner side of membrane was buffered with 8 mM EGTA to 0.01 μM, calculated using 'Maxc Software' (from Chris Patton, Hopkins Marine Station, Stanford University).

Results are expressed as means ± standard deviation where appropriate. Each experiment was repeated several times. Student's *t*-test was used for statistical comparison among means, and differences with $P < 0.05$ were considered significant.

2.5. Computer analysis

Nucleic acid and protein sequences were compared with sequences in the GenBank databases using program BLAST. The 'Search the Conserved Domain Database using RPS-BLAST' (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) program has been used for search of conservative domains. The sequences of the open reading frames of the *KCNRG* and *KCNRG*-like genes were aligned, using the ClustalW program (www2.ebi.ac.uk/ClustalW/).

3. Results and discussion

3.1. Molecular cloning of a novel putative tumor suppressor gene KCNRG

To identify the candidate for tumor suppressor genes for BCLL and prostate cancer locus at 13q14.3, an integrated genomic contig [8] and corresponding transcript map [9] were constructed. The new gene *KCNRG* was found via BLASTN search of the EST database of GenBank seeded with a genomic sequence (AF440619). As a result we retrieved two expressed human sequences with the accession numbers BI770863 and BG537776. Corresponding cloned cDNAs IMAGE: 5207702 and IMAGE: 4690662 were completely sequenced (mRNAs AY129654 and AY129653) and found to contain two full-length coding sequences corresponding to different mRNA isoforms that belongs to the same gene.

Sequence alignment of the genomic locus AF440619, which consists of 350 000 bases with the *KCNRG* mRNAs AY129654 and AY129653 revealed genomic structure of 5460 bp *KCNRG*. mRNA AY129654 consists of two exons (612 nt and 709 nt in size) divided in genome by single intron 4139 nt in size, mRNA AY129653 consists of three exons (612, 95 and 709 nt in size) divided by two introns (2750 and 1292 nt in size). All of the intron–exon junctions conformed to the consensus splice sites, GT and AG, for donor and acceptor sequences, respectively (data not shown). The mRNA isoforms AY129654 and AY129653 result from alternative splicing events (Fig. 1).

3.2. Analysis of amino acidic sequence encoded by gene KCNRG

As the newly found gene encodes a protein with strong amino acidic sequence similarity to cytoplasmic tetramerization domain (T1) of voltage-gated K^+ channels, we assigned to this gene a name *KCNRG* (K^+ channel regulator) and

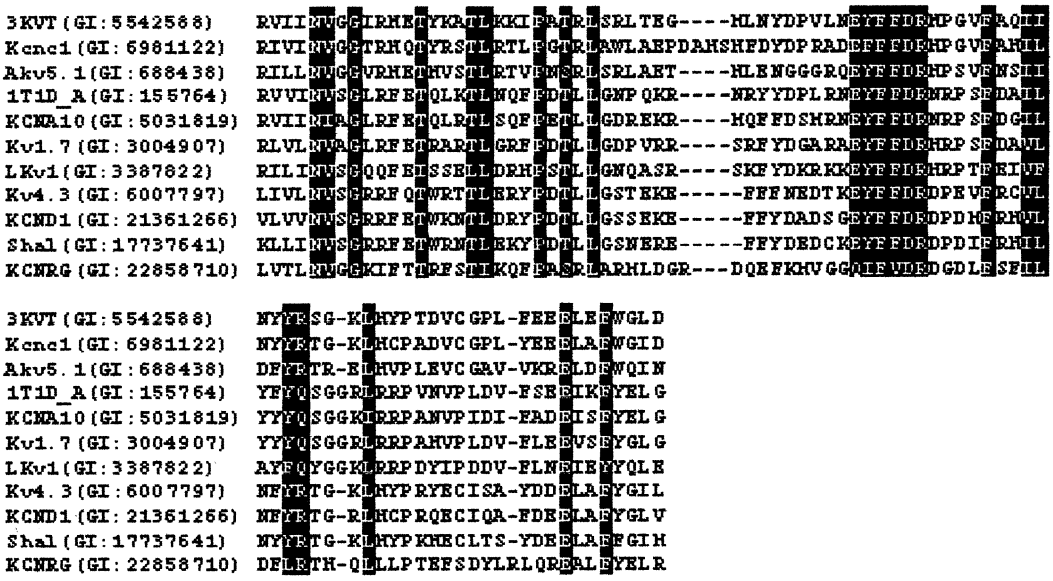

Fig. 2. Alignment of KCNRG to selected proteins containing of T1 domain. Black boxes indicate residues that are conserved among 247 primary sequences from GenBank.

registered the mRNA isoforms as AY129654 (1345 nt encoding 272 aa) and AY129653 (1465 nt encoding 229 aa). Hydrophilicity analysis of the amino acid sequences encoded by mRNAs AY129654 and AY129653 showed that both of them are soluble proteins that differ only in their C-terminal amino acidic sequences. The N-termini are identical in their amino acidic sequences and both isoforms show strong homology (*e*-value 8*e*–14) to T1 domain (pfam 02214) of voltage-gated K^+ channels (Fig. 2) and to BTB/POZ domain with considerably lower *e*-value equal 3.90*e*–06. T1 domain of voltage-gated K^+ channels encodes molecular determinants for subfamily-specific assembly of α-subunits into functional tetrameric channels. A presence of T1 domain structure in the KCNRG suggested that protein might interfere with normal assembly of the K+ channel proteins and their tetramerization thus causing the suppression of the K+ channels.

3.3. *Tissue distribution of* KCNRG *mRNA isoforms*

RT-PCR analysis for human multiple mRNA showed that *KCNRG* mRNA was expressed in normal and some tumor tissues (Fig. 3). With 35 cycles of PCR electrophoretically visible bands corresponding to both alternatively spliced mRNA isoforms of the gene *KCNRG* were detected in normal lung, lymphocytes, prostate and tumor cell lines human colon carcinoma (Lim 1215), human epidermal carcinoma (A431), human breast cancer (T47D) and human osteogenic sarcoma (SAOS-2). *KCNRG* was expressed highly in normal lung. With 39 cycles of PCR DNA bands of the same weight were visible also in the normal kidney, stomach, brain, but not in the tumor cell lines of metastatic prostate carcinoma (LNCaP), ovarian epithelial carcinoma (SKOV-3), osteosarcoma (T_{1-13}).

3.4. *Electrophysiological study protein* KCNRG

To test the hypothesis that the KCNRG protein is able to interfere with the normal function of the K^+ channels (see above), we cloned *KCNRG* sequence in to pEGFP-N1 vector and used this construction to express KCNRG-GFP in LNCaP human prostate derived cell culture. In the experiments with the whole-cell patch-clamp technique potassium current was measured in 38 transfected and in 32 non-transfected LNCaP cells. The average K^+ current density in transfected cells (78.9 ± 26.2 pA/pF, $n = 38$) was two times lower than in the control cells (164.8 ± 38.7 pA/pF, $n = 32$) as shown in Fig. 4A. It is difficult to compare K^+ currents directly in the individual transfected and non-transfected cells because of the very broad diversity of individual K^+ currents. To overcome this problem we focused on symmetrical recently divided cell pairs. Indeed such pairs had rather close values of K^+ current (17 ± 8% difference, $n = 10$) and practically the same membrane capacitance, although K^+ current alone varied strongly between different pairs (between 80 and 196 pA/pF). We investigated four cases where one cell from pair was transfected while the second one was not transfected (Fig. 4B). In all these cases, K^+ currents were obviously lower in transfected cells than in non-transfected ones. Fig. 4C,D shows an example of the family of K^+ currents (C) obtained

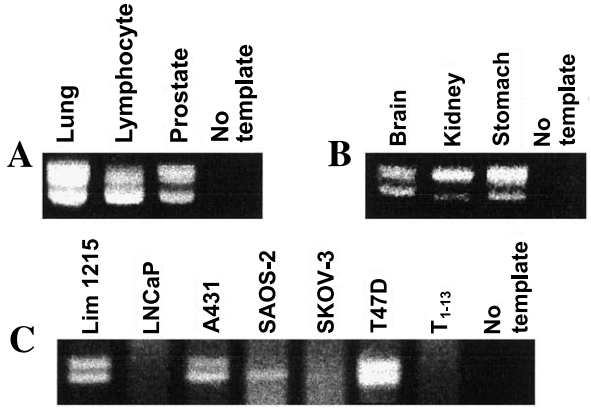

Fig. 3. Tissue distribution of isoforms mRNA gene *KCNRG*. A: RT-PCR analysis for human multiple mRNA with 35 cycles; B: RT-PCR analysis for human multiple mRNA with 39 cycles; C: Total RNA from cultures of tumor cells were used for RT-PCR of *KCNRG* with 35 cycles. As the positive control of these RT-PCR reactions, *GAPDH* was amplified (data not shown).

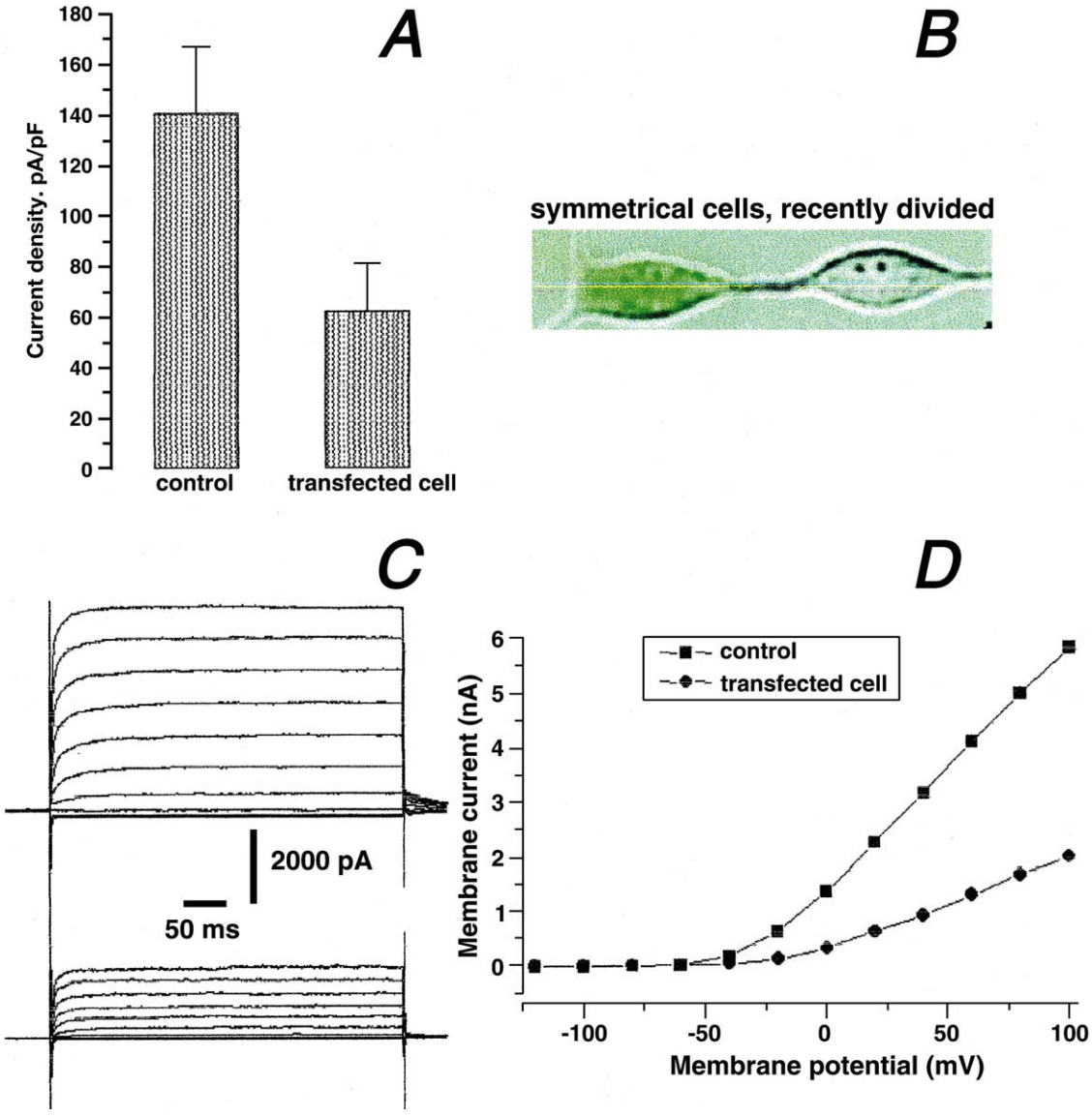

Fig. 4. The expression of KCNRG in LNCaP cells is suppressing K^+ currents. A: Summary histogram of K^+ current density in control and KCNRG-transfected cells. B: Example of paired symmetrical cells, one of them is transfected while other is not transfected. C: K^+ currents in paired non-transfected (top; membrane capacitance 20 pF) and transfected (bottom; membrane capacitance 17 pF) cells. Holding potential was −40 mV; testing potentials were from −120 to 100 mV. D: Voltage–current relations of non-transfected and transfected cells. Data are taken from C.

in transfected and control non-transfected LNCaP cells evoked by 400 ms depolarizing pulses from a holding potential of −80 mV to various membrane potentials (−120 to +100 mV) and the corresponding current–voltage relationship (D).

In control experiments with LNCaP cells transfected with EGFP alone no difference was detected in K^+ currents between transfected ($N = 11$) and non-transfected ($N = 37$) cells (data not shown).

3.5. Summary

In this report, we describe the cloning of *KCNRG*, a novel K^+ channel regulator encoding gene, which was identified by human genome sequence database search for a new genes located in 13q14.3. *KCNRG* gene contains three exons, including one that can be spliced producing two alternative mRNA isoforms. This gene is ubiquitously expressed since it was present in all normal and some tumor tissues analyzed.

KCNRG encodes a soluble protein. Analysis of protein sequence databases revealed a significant homology between the KCNRG N-termini amino acid sequence and the domain T1 of voltage-gated K^+ channels. Electrophysiological study on LNCaP cell reveals that KCNRG protein expression inhibits K^+ fluxes.

As voltage-gated K^+ channels are upregulated in immature neoplastic BCLL cells and as their selective blockers suppress lymphocyte and LNCaP human prostate cancer cell lines proliferation, we suggest that *KCNRG* is a candidate for a BCLL and prostate cancer tumor suppressor gene in human 13q14.3.

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