Primary cilium, cortactine, and Kv10.1 dynamics under confocal microscopy

ESTUDIO DE LA DINÁMICA DEL CILIO PRIMARIO Y DEL CANAL DE POTASIO Kv10.1 MEDIANTE MICROSCOPÍA CONFOCAL

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Arch. Med. Univ. 2016, Nº4, ISNN: 2341-0361.
Reception date: 31/07/2015
Acceptance date: 14/09/2015

OBJECTIVES: IT IS BELIEVED THAT TUMOR CELLS EXPRESSING Kv10.1 CHANNEL ACQUIRE SELECTIVE ADVANTAGES THAT ALLOW THEM TO MAINTAIN CHRONIC PROLIFERATION. THE CILIARY DISASSEMBLY IS A PREREQUISITE TO ENTER INTO THE CELL CYCLE, THEREFORE OUR OBJECTIVE WAS TO FIND CORRELATIONS BETWEEN PRIMARY CILIUM DISASSEMBLY AND THE Kv10.1 CHANNEL THROUGH THE Kv10.1 CORTACTIN BINDING SITE.

METHODS: WILD TYPE Kv10.1, AS WELL AS THE MUTANT FORM LACKING THE BINDING SITE TO THE CYTOSKELETON PROTEIN CORTACTIN, WERE OVEREXPRESSED IN RPE-TERT CELL LINE. THE EFFECT ON CILIARY DISASSEMBLY OF SUCH OVEREXPRESSION FORMS WAS IMAGING USING CONFOCAL MICROSCOPY.

RESULTS: Kv10.1 WILD TYPE OVEREXPRESSION INDUCED DISASSEMBLY OF THE PRIMARY CILIUM AND ALTERATION OF THE CYTOSKELETON, WHICH CORRESPONDED TO OVERPRODUCTION OF FILAMENT STRUCTURES POSITIVE TO α-TUBULIN ACETYLATED IN THE CYTOPLASM. OVEREXPRESSION OF MUTATED Kv10.1 DID NOT SHOW THIS PATTERN, NOR THE UNTREATED CELLS.

CONCLUSIONS: THIS DATA SUGGESTS THAT Kv10.1 CHANNEL COULD BE INTERFERING WITH THE CILIUM FORMATION, AND THUS INFLUENCES THE CELL CYCLE IN A WAY THAT CORTACTINE BINDING SITE IS NOT INVOLVED. THAT SHOULD BE FURTHERLY STUDIED BECAUSE DUE TO ITS POSSIBLE IMPLICATIONS IN CANCER.

KEYWORDS: Kv10.1 CHANNEL, PRIMARY CILIUM, CORTACTIN, ACETYLATED ALPHA-TUBULIN.

PALABRAS CLAVE: CANAL Kv10.1, CILIO PRIMARIO, CORTACTINA, ALFA-TUBULINA ACETILADA.

Introduction and objective

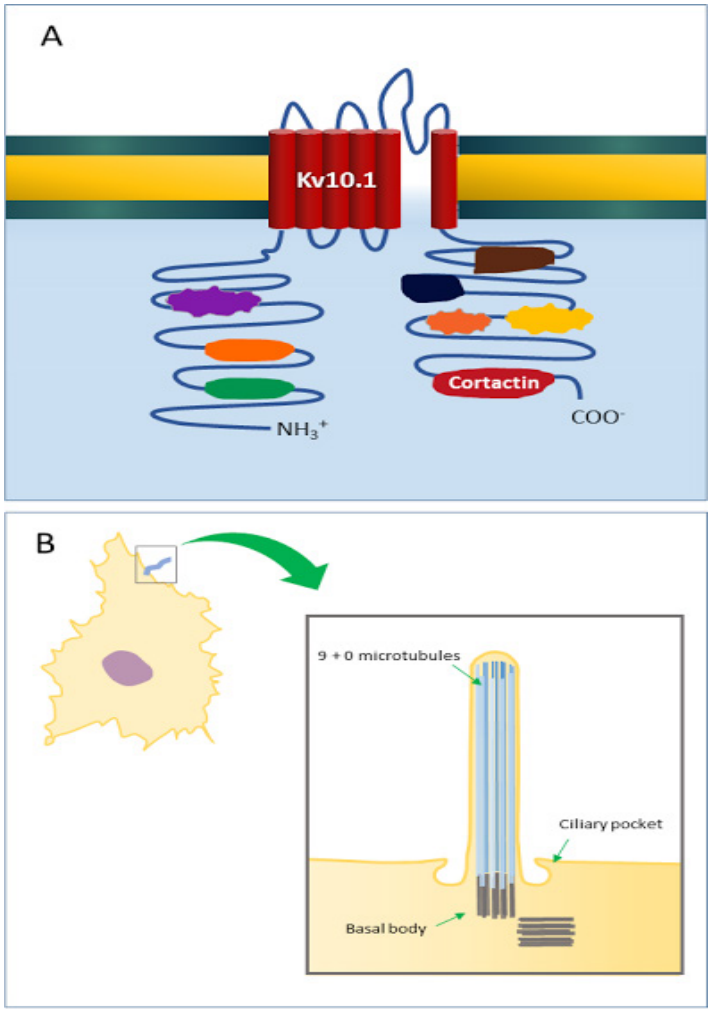
Kv10.1, also known as Eag1 (Ether-à-go-go-1) is encoded by the KCNH1 gene, and belongs to the KCNH family of voltage-dependent potassium channels (1). A striking feature of Kv10.1 is its widespread presence in tumor biopsies and somatic cancer cell lines, despite being preferentially expressed in brain among normal tissues (2) (3). Indeed, approaches taking advantage of its membrane localization and preferential expression in cancer cells have been successfully tested *in vitro*. Using an anti-Kv10.1 antibody coupled to TRAIL (TNF-related apoptosis-inducing ligand), cancer cells are selectively induced to undergo apoptosis (4).

Nevertheless, the mechanisms of how Kv10.1 favors cell proliferation and enhances tumor progression are poorly understood. It is known that hEAG channels, as Kv10.1, establish complex interactions with cytoskeletal elements, and that these interactions strongly influence the properties of the channels (5). As visualized in Image 1-A, Kv10.1 channel has a cortactin binding site, a cytoskeletal architecture protein regulator which is amplified in several types of cancer (6). The effect of the union between cortactin and its Kv10.1 binding site remains unclear, but it might affect primary cilium dynamics (Figure 1A).

The primary cilium is a microtubule-based membranous protrusion found in almost all cell types that functions as a sensory center that regulates cell proliferation and embryonic development (7). Defects of primary cilia and their related proteins cause the dysregulation of cell proliferation and embryonic development (8). The primary ciliary pocket is a specialized endocytic membrane domain in the basal region. The basal body of a primary cilium exists as a form of the centriole during interphase of the cell cycle. Although conventional thinking suggests that the cell cycle regulates centrosomal changes, recent studies suggest the opposite, that is, centrosomal changes regulate the cell cycle (7) (Figure 1B).

The ciliary pocket connects to the actin cytoskeleton and probably serves as an interface between the primary cilium and the actin cytoskeleton. The actin cytoskeleton and the basal body region also interact and this interaction might participate in the migration of the basal body to the apical cell membrane and consequently in the cell division (9) (10).

Then, Kv10.1 presents a cortactin binding site, which also binds to cytoskeletal actin and influences primary cilium dynamics, which may be related to mitosis entrance, and so with cancer. We investigate the effects of mutations in the cortactin binding site of Kv10.1 in primary cilium presence.



▲ **Figure 1**. *(A) Graphic representation of Kv10.1 channel, with its intracytoplasmic domains. Cortactin binding site is represented. (B) Primary cilium is shown with its 9+0 microtubular structure, as well as the basal body (centrosome) and the ciliary pockets.*

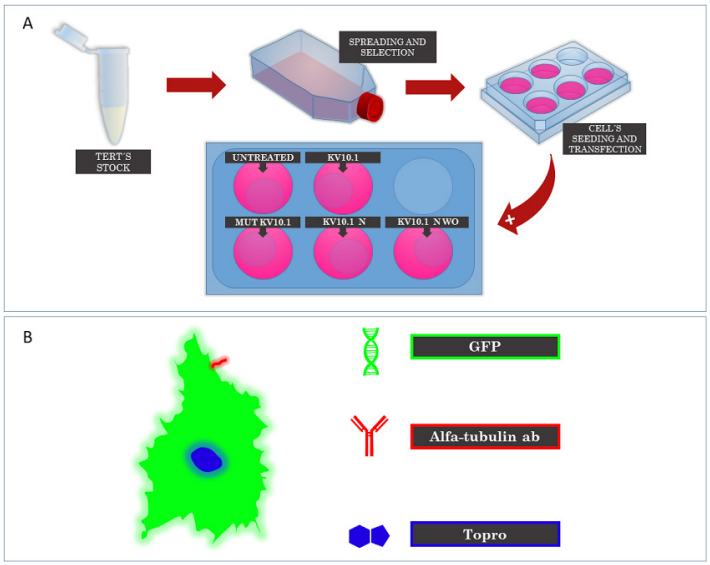
Materials and methods

Cell culture and transfection

We used hTERT RPE-1 cell lines (human telomerase reverse transcriptase retinal pigment epithelial cell line 1), which were grown in Dulbecco's modified Eagle's medium (DMEM) with hygromycin to select the immortalized cells. Then, about 100.000 cells were attached to coverslips using fibronectin, 20 microliters per well.

The cell culture of hTERT RPE-1 line was carried out under five different conditions: untreated; transfected with wild type (WT) Kv10.1; transfected with mutated Kv10.1 in the binding site of the cytoskeletal protein cortactin; transfected with WT Kv10.1 treated with nocodazole 1mM (an antineoplastic agent which interferes with the polymerization of microtubules); and transfected WT Kv10.1 treated with nocodazole and wash out with DSMO.

▼ **Figure 2**. *(A) Representation of the steps to do the culture and transfections. Cells are defrosted and cultivated, being expanded afterwards in flasks. After cell trypsinization, cells are cultivated in wells containing coverslips and lipofectamine transfection is done. Conditions: control, transfected with WT Kv10.1; transfected with WT Kv10.1 treated with nocodazole; transfected with WT Kv10.1 treated with nocodazole and washout; and transfected with MUT Kv10.1. (B) Representation of a cell stained with immunocytochemistry techniques in confocal microscopy (left) and the different immunofluorescent molecules used (right).*



WT and mutated Kv10.1 were transfected into hTERT RPE-1 cells using Lipofectamine LTX Reagent with Plus Reagent, according to Life-Technologies' protocol. Stable transfections were selected by the expression of GFP (Figure 2A).

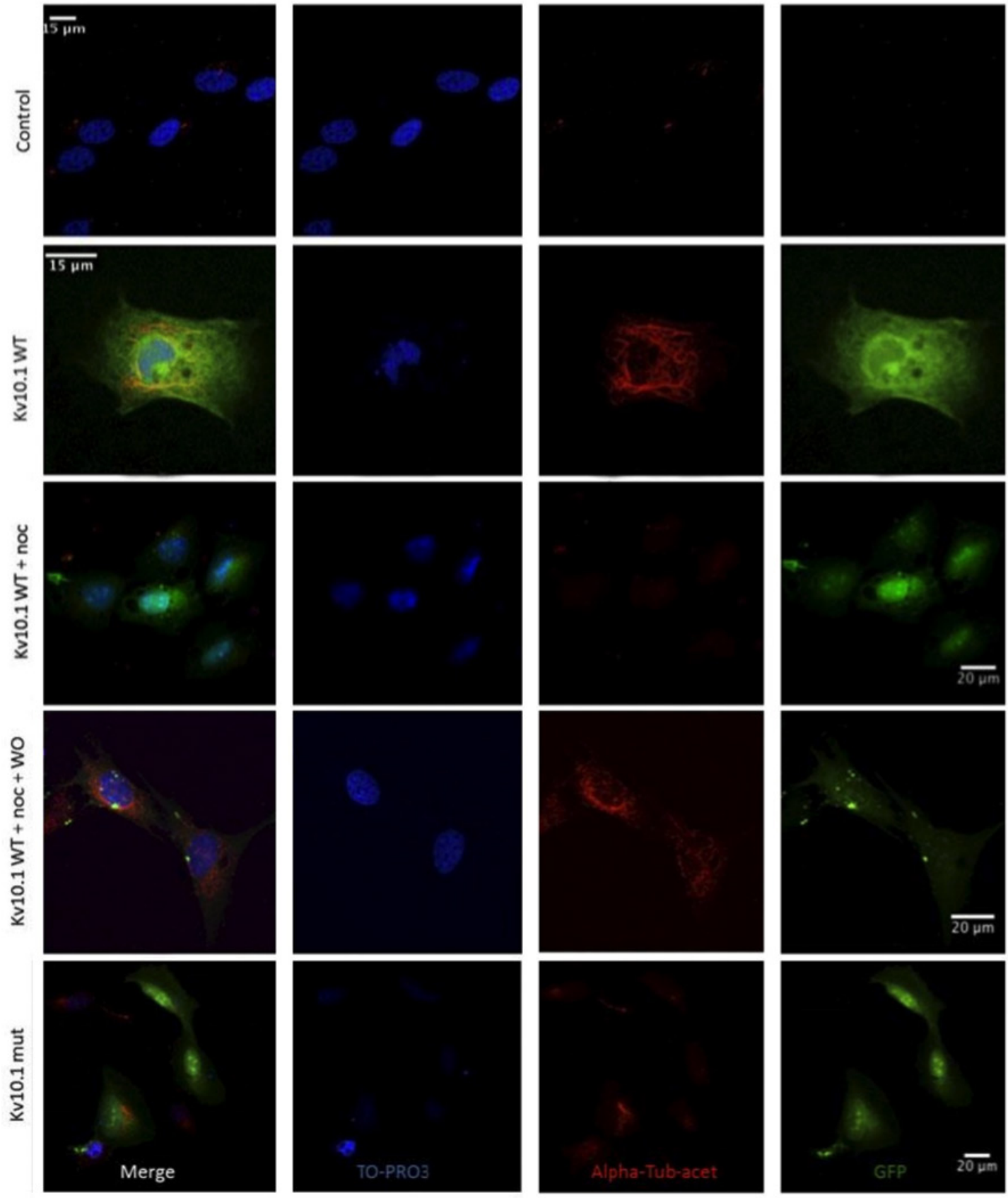
Immunocytochemistry

Fifteen coverslips of cells were fixed using 10 % formalin solution at 4 °C for 10 minutes, washed 3 times with PBS, and permeabilized with 0.5 % Triton X-100 in PBS for 5 minutes. Then, cells were washed 3 times with PBST (0.05 % Tween 20 in PBS), blocked with 10 % BSA in PBST for 1 hour, and incubated with the primary antibody against α-acetylated tubulin (ab24610-mouse, dilution 1:2000) overnight at 4 °C.

Afterwards, cells were rinsed 3 times, incubated for 1 hour at room temperature with the secondary antibody Alexa Fluor 546 goat anti-mouse (dilution 1:1000), and washed again 3 times. Finally, the nuclei were stained with 1:1000 dilution of TO-PRO-3.

All the procedures were performed following the protocol provided by Dr. Urrego (11).

Cells were imaged with confocal laser scanning microscopy, using Zeiss LSM 510 Meta and ZEN software. We used the 63x oil immersion objective and excitation with HeNe1 Laser for alpha acetylated tubulin and Argon Laser for GFP (Figure 2B).



▲ **Figure 3**. *Representative images of control cells; transfected with WT Kv10.1, transfected with WT Kv10.1 and treated with nocodazole; transfected with WT Kv10.1 treated with nocodazole and washout; and transfected with Kv10.1 mutated in the binding site of cortactin (MUT). They are stained with anti-alpha-tubulin acetylated antibodies (red). Transfected cells express GFP (green). Nuclei are stained with TO-PRO 3 (blue). Control cells present a primary cilium, which is positive to acetylated alpha-tubulin. WT Kv10.1 expression shows a cytoplasmic structure positive to acetylated alpha tubulin. This structure is dissolved with nocodazole and reappears when it is washed out, which suggests that the structure is not an artefact, but a microtubule made structure. MUT Kv10.1 expression leads to a microtubules' deficient stabilization and does not show a primary cilium.*

Results

The images obtained, which can be seen in figure 3, show that untreated cells presented primary cilium, which is seen as a red filamentous structure stained with alpha-tubulin acetylated. As expected, they do not express GFP, as they were not transfected.

Nonetheless, cells with WT Kv10.1 overexpression are stained with GFP. This overexpression induced an alteration of the cytoskeleton, which is visualized as an overproduction of filamentous structures positive to alpha-tubulin acetylated in the cytoplasm. This prevents us from determining the presence of the primary cilium. The addition of nocodazole, which disassembles the microtubules, led to a disappearance of the structure, whereas the washout of nocodazole resulted in the reappearance of the alpha-tubulin acetylated structure, thus suggesting that it is not a mere artefact.

Finally, MUT Kv10.1 overexpression did not show the alpha-tubulin acetylated structure, nor a clear primary cilium.

Discussion

In cells with Kv10.1 overexpression, the visualized overproduction of filamentous structures positive to alpha-tubulin acetylated in the cytoplasm prevents us from determining the presence of the primary cilium. Further studies are needed to determinate the presence of the primary cilium in cells with WT Kv10.1 overexpression. This could be made by staining the centrosomes in order to determinate their position in the cell, as primary cilium has always a centrosome in its base.

The fact that MUT Kv10.1 overexpression did not show the alpha-tubulin acetylated structure, nor a clear primary cilium, may indicate that cortactin is responsible for the formation of the alpha-tubulin acetylated structure seen in cells with WT Kv10.1 overexpression. The loss of microtubules stabilization cortactin's function may prevent its formation. Interaction between cortactin and Kv10.1 may not be responsible of the ciliary disassembly, since its impaired union to Kv10.1 channel does not promote ciliary formation.

Conclusions

These results suggest that Kv10.1 channel could be interfering with the cilium formation in a way in which cortactin is not involved. Therefore, Kv10.1 channel cortactin binding site would not play a determining role at influencing the cell cycle and increasing proliferation.

Further research on Kv10.1 pathways and its interactions with cytoskeleton and primary cilium will give us better understanding of such fundamental processes as cell cycle and proliferation. This may have therapeutic implications, as they are related to cancer, and may improve the design of new antineoplastic agents specifically against these mechanisms.

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