Bidirectional KCNQ1:β-catenin interaction drives colorectal cancer cell differentiation

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The K+ channel KCNQ1 has been proposed as a tumor suppressor in colorectal cancer (CRC). We investigated the molecular mechanisms regulating KCNQ1:β-catenin bidirectional interactions and their effects on CRC differentiation, proliferation, and invasion. Molecular and pharmacologic approaches were used to determine the influence of KCNQ1 expression on the Wnt/β-catenin signaling and epithelial-to-mesenchymal transition (EMT) in human CRC cell lines of varying stages of differentiation. The expression of KCNQ1 was lost with increasing mesenchymal phenotype in poorly differentiated CRC cell lines as a consequence of repression of the KCNQ1 promoter by β-catenin:T-cell factor (TCF)-4. In well-differentiated epithelial CRC cell lines, KCNQ1 was localized to the plasma membrane in a complex with β-catenin and E-cadherin. The colocalization of KCNQ1 with adherens junction proteins was lost with increasing EMT phenotype. ShRNA knock-down of KCNQ1 caused a relocalization of β-catenin from the plasma membrane and a loss of epithelial phenotype in CRC spheroids. Overexpression of KCNQ1 trapped β-catenin at the plasma membrane, induced a patent lumen in CRC spheroids, and slowed CRC cell invasion. The KCNQ1 ion channel inhibitor chromanol 293B caused membrane depolarization, redistribution of β-catenin into the cytosol, and a reduced transepithelial electrical resistance, and stimulated CRC cell proliferation. Analysis of human primary CRC tumor patient databases showed a positive correlation between KCNQ1:KCNE3 channel complex expression and disease-free survival. We conclude that the KCNQ1 ion channel is a target gene and regulator of the Wnt/β-catenin pathway, and its repression leads to CRC cell proliferation, EMT, and tumorigenesis.

KCNQ1 | β-catenin | colon cancer | epithelial-mesenchymal transition | adherens junctions

The development of colorectal cancer (CRC) is determined by multiple factors including ion transport (1, 2). During the last 10 years, evidence for the role of K+ channels in the development and growth of tumors has greatly expanded. Voltage-gated K+ channels (Kv) are involved in the proliferation of many cell types, including intestinal cells. Although the recent literature clearly demonstrates that Kv channels are among the targets of interest in the fight against cancer (3–5), the specific role of each Kv channel in tumorigenesis and the molecular mechanisms involved are unknown. This is notably the case of the KCNQ1 K+ channel. The KCNQ1 gene has recently been identified as a tumor suppressor in mouse and human CRC tissues (6). KCNQ1 deficiency in mice caused rectal adenomatous hyperplasia and progression to adenocarcinoma. A loss of imprinting of KCNQ1 has been described in CRC (7). However, the functional and molecular events linking KCNQ1 and CRC progression remain unclear. One obvious pathway, which may interact with KCNQ1, is Wnt/β-catenin signaling, which plays a key role in driving early embryogenesis, as well as intestinal homeostasis and stem cell renewal in the intestinal mucosa epithelia (8). Deregelation of the β-catenin signaling axis is present in more than 80% of CRCs. This can lead to β-catenin accumulation in the cytosol, increased nuclear translocation of activated β-catenin, interactions with members of the T-cell factor (TCF) family, and stimulation of β-catenin-dependent gene expression, leading to increased cell proliferation and growth (9). In CRC cells, excessive nuclear accumulation of β-catenin was shown to increase the transcription of KCNQ1OT1 (10), a long noncoding RNA known to negatively regulate KCNQ1 expression. A possible link between β-catenin and ion channels in CRC has never been established. In this study, we demonstrate a bidirectional interaction between KCNQ1 and β-catenin regulating CRC cell differentiation processes. We also demonstrate that KCNQ1 is a target gene of β-catenin:TCF4, and that the expression, as well as the ion channel function, of KCNQ1 module epithelial phenotype. Moreover, KCNQ1 expression and its regulatory channel subunit KCNE3 (11) were correlated with better CRC patient survival.

Results

Loss of KCNQ1 Is Associated With Mesenchymal Phenotype in CRC Cells. In normal colonic epithelium, KCNQ1 drives Cl− secretion and generates the resting membrane potential (12, 13). Low expression of KCNQ1 has been correlated with CRC development in APC mouse models, but the function of the channels remains unknown (6). Using Western blotting, we compared the expression of KCNQ1 protein in six CRC cell lines of varying differentiation states and observed a high expression in

Significance

The K+ channel KCNQ1 has been proposed as a tumor suppressor in colorectal cancer (CRC), but nothing is known about its regulatory role in early disease stages. KCNQ1 is a target gene of Wnt/β-catenin, which is tonically activated in CRC. We demonstrate a bidirectional interaction between KCNQ1 and β-catenin as a key regulator of CRC cell differentiation, proliferation, and invasion. KCNQ1 stabilizes β-catenin at adherent junctions to maintain an epithelial phenotype. The β-catenin: T-cell factor (TCF)-4 transcriptional pathway directly represses KCNQ1 expression, and the loss of KCNQ1 was associated with an epithelial–mesenchymal transition. The KCNQ1:KCNE3 ion channel complex expression in primary tumors was correlated with good survival outcome for patients with CRC. KCNQ1 is a potential early prognostic biomarker for CRC.


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The activation of GSK3 increased using two inhibitors of glycogen synthase kinase 3 expression, using a pharmacologic approach. Well-differentiated cells (HT29cl.19A and HT29) and a low expression is moderate to poorly differentiated cells (DLD-1, HCT116, SW480, SW620) (Fig. 1A). This observation was confirmed by quantitative PCR showing a decreasing expression of KCNQ1 mRNA with increasing mesenchymal phenotype (Fig. 1B). A high KCNQ1 expression was associated with strong expression of the epithelial marker E-cadherin and low expression of the mesenchymal marker N-cadherin. The converse was also found: a low expression of KCNQ1 was associated with a low expression of E-cadherin and a high expression of N-cadherin (Fig. 1A). Thus, it appears that KCNQ1 expression decreases with epithelial-to-mesenchymal transition (EMT) differentiation and in isolated rat colonic crypts. In the well-differentiated HT29cl.19A cells, as well as in rat crypt cells, the expression of KCNQ1 was colocalized with β-catenin at the plasma membrane (Fig. 1C and D). In contrast, KCNQ1 was undetectable in the mesenchymal phenotypic SW480 cells, which showed high abundance of β-catenin in the cytosol (Fig. 1C). The colocalization between KCNQ1 and β-catenin was confirmed by van Steensel’s analysis (14) in both HT29cl.19A cells and crypt cells in which a similar Pearson’s coefficient value was found (SI Appendix, Fig. S1 A and B).

Activation of β-Catenin Causes Direct Transcriptional Repression of KCNQ1. We investigated the role of β-catenin in KCNQ1 expression, using a pharmacologic approach. β-catenin activity was increased using two inhibitors of glycogen synthase kinase 3β (GSK3β): AR-A014418 (AR-A) and GSK3-inhibitor X (GSK3-X). The activation of β-catenin and its translocation into the nuclear fraction after inhibition of GSK3β was verified in HT29cl.19A cells (SI Appendix, Fig. S2). Activation of β-catenin reduced KCNQ1 total abundance in HT29cl.19A cells by 60% (Fig. 2A) and reduced the colocalization of KCNQ1 with β-catenin at the plasma membrane (SI Appendix, Fig. S3). Similar effects of pharmacologic agents were observed in HT29 cells (SI Appendix, Fig. S4). In addition to the pharmacologic approach, we also used a plasmid expressing a truncated form of β-catenin (β-catΔN87) to constitutively activate β-catenin. HT29cl.19A and HT29 cells transfected with activated β-catenin showed a loss of KCNQ1 and a redistribution of β-catenin from the plasma membrane into the cytosol (SI Appendix, Fig. S5). Chromatin immunoprecipitation (ChIP) and luciferase assays were performed to determine the effects of β-catenin activation on KCNQ1 transcription. We chose SW480 cells because of their high constitutive expression of β-catenin. Treatment with GSK3-IX to increase β-catenin activation resulted in enhanced recruitment of β-catenin and TCF4 proteins to the KCNQ1 promoter (Fig. 2 B and C). Furthermore, the luciferase assay showed that the KCNQ1 reporter transcriptional response was reduced in Cho cells treated with GSK3-IX compared with vehicle-treated control (Fig. 2D). To confirm the role of TCF4 in the control of KCNQ1 expression, DLD-1 and SW480 cells, which show low constitutive KCNQ1 expression, were transfected with a plasmid carrying a dominant negative mutant of TCF4 (hΔNCTF4), which cannot form a complex with β-catenin. This experiment revealed that the expression of KCNQ1 was restored in the presence of hΔNCTF4 in both cell lines (Fig. 2E and F). Taken together, these data provide a molecular mechanism by which the activated Wnt/β-catenin pathway inhibits KCNQ1 protein expression via direct binding of a repressive β-catenin:TCF4 complex at the KCNQ1 promoter.

KCNQ1 Modulates β-Catenin Interactions at Adherens Junctions. In situ proximity ligation assay (PLA) revealed that KCNQ1 binds to both E-cadherin and β-catenin, which are markers of adherens junctions (AJ). In rat colonic crypts, KCNQ1 and β-catenin were observed by PLA to be in close association (Fig. 3A and SI Appendix, Fig. S6). PLA dots were mainly observed at cell-to-cell contacts, and this was verified in HT29cl.19A cells, showing that KCNQ1 is a component of AJ in close contact (<40 nm) with β-catenin and E-cadherin (Fig. 3B and SI Appendix, Fig. S6).
Furthermore, silencing of KCNQ1 in HT29cl.19A cells using ShRNAs resulted in a significant disruption to E-cadherin-β-catenin interactions (Fig. 3C). These observations indicate the importance of KCNQ1 in maintaining AJ stability and tight junction formation. This was further demonstrated by the reduction in transepithelial electrical resistance (TEER) in HT29cl.19A cells in HT29cl.19A cells. Nuclear DAPI staining is in blue (n = 3). (Scale bars, 15 μm.) PLA showing interaction between β-catenin and E-cadherin in HT29cl.19A stably expressing a nontargeting ShRNA (ShRD) or a ShRNA targeting KCNQ1 mRNA (ShQ1-1). (Scale bars, 15 μm.) PLA dots quantification is shown on the bar graph (n = 3; *P < 0.05). (D) TEER in ShRNA KCNQ1 (ShQ1-1; ShQ1-2) HT29cl.19A monolayers (n = 6, ***P < 0.001). Insert shows the protein expression of KCNQ1 by Western blot analysis. (E) Western blot and densitometry analysis showing the effect of the molecular silencing of β-catenin residues that determine its subcellular distribution and stability. The level of p-S33 phosphorylation that induces the degradation of cytosolic β-catenin (15, 16) was lower in the KCNQ1 knockdown cell lines (Fig. 3E). In contrast, phosphorylation at residues Y654 and S675, which favor the stabilization of cytosolic β-catenin and its translocation into the nucleus, was significantly increased in KCNQ1-silenced cells (Fig. 3E). These results lend strong support for the conclusion that KCNQ1 is a pivotal regulator of β-catenin stability and subcellular distribution. As a corollary, the data indicate that KCNQ1 suppresses the Wnt/β-catenin signaling pathway. This was indeed shown to be the case where silencing KCNQ1 expression activated signaling intermediates of the Wnt/β-catenin pathway, increasing the expression of p-akt, p-gsk-3β, cyclin d-1, cJun, and Met (SI Appendix, Fig. S7 A and C).

**Fig. 3**. KCNQ1-β-Catenin and E-cadherin interactions at the plasma membrane. (A) PLA showing interaction (red signal) between KCNQ1 and β-catenin in isolated rat colonic crypts (12 crypts from 3 rats). The negative control lacking primary antibodies is shown. Nuclear DAPI staining is in blue. (Scale bars, 10 μm.) (B) PL A showing interaction (red signal) between KCNQ1 and β-catenin and also between KCNQ1 and E-cadherin in HT29cl.19A cells. Nuclear DAPI staining is in blue (n = 3). (Scale bars, 15 μm.) (C) PLA showing interaction between β-catenin and E-cadherin in HT29cl.19A stably expressing a nontargeting ShRNA (ShRD) or a ShRNA targeting KCNQ1 mRNA (ShQ1-1). (Scale bars, 15 μm.) PLA dots quantification is shown on the bar graph (n = 3; *P < 0.05). (D) TEER in ShRNA KCNQ1 (ShQ1-1; ShQ1-2) HT29cl.19A monolayers (n = 6, ***P < 0.001). Insert shows the protein expression of KCNQ1 by Western blot analysis. (E) Western blot and densitometry analysis of different phosphorylated β-catenin residues in HT29cl.19A KCNQ1 knockdown cells (n = 3–5). *P < 0.05; **P < 0.01; ***P < 0.001).

KCNQ1 Expression Suppresses Mesenchymal Features in CRC Spheroids. Knock-down of KCNQ1 induced a significant increase in mesenchymal markers (claudin-1, β-catenin, and N-cadherin), with a concomitant reduction in epithelial markers (ZO-1 and E-cadherin) (Fig. 4A). When grown on a 3D matrix, highly differentiated CRC cells such as HT29cl.19A underwent cystogenesis and generated spheroid structures. HT29cl.19A spheroids showed characteristic accumulation of F-actin at the apical membrane, β-catenin lateral staining, a patent central lumen, and well-defined adherens junctions (Fig. 4B). Knock-down of KCNQ1 significantly impaired the ability of HT29cl.19A cells to form spheroids, and the frequency of lumen formation was reduced from 80% to 20% (Fig. 4B).

We examined the effects of overexpression of KCNQ1 in an intermediate EMT cell line HCT116 with low endogenous expression levels of KCNQ1. KCNQ1 construct expression in HCT116 cells led to an increase in epithelial markers (ZO-1 and E-cadherin) and a decrease in mesenchymal markers (β-catenin and N-cadherin) (Fig. 5A). The overexpression of KCNQ1 in HCT116 cells restored the ability of this moderately differentiated CRC cell line to form spheroid structures, and the frequency of lumen formation was increased from 19% to 62% (Fig. 5B). Collectively, these results demonstrate that KCNQ1 is required to maintain a well-differentiated epithelial phenotype.

**Fig. 4**. KCNQ1 expression and epithelial cell phenotype. (A) Western blot and densitometry analysis showing the effect of the molecular silencing of KCNQ1 on the expression of epithelial and mesenchymal markers in HT29cl.19A control cells (ShRD) or in HT29cl.19A ShKCNQ1 (ShQ1-1 and ShQ1-2). Bar graph shows protein expression normalized to tubulin (n = 3–5). *P < 0.05; **P < 0.01; ***P < 0.001. (B) Confocal immunofluorescence images of HT29cl.19A (ShRD) and KCNQ1 knock-down cells spheroids grown in Matrigel showing β-catenin (green), F-actin (red), and nuclear staining (DAPI-blue). (Scale bar, 20 μm.)
KCNQ1 expression (SI Appendix, Fig. S10). These data demonstrate that KCNQ1 channels act to repress proliferation and invasion of CRC cells.

The KCNQ1 Channel Complex Is Associated with CRC Patient Survival. The KCNQ1 functional channel complex in colon is composed of the KCNQ1 pore-forming unit and a KCNE3 regulatory subunit. The KCNQ1 functional channel complex in colon is composed of the KCNQ1 pore-forming unit and a KCNE3 regulatory subunit. In a cohort of 355 human primary colon tumor samples, Kaplan Meier and log rank test analysis demonstrated that CRC relapse-free survival was significantly higher for patients with high KCNQ1 (Fig. 7A) and high KCNE3 mRNA expression (Fig. 7B). TCF4, which we have shown to suppress the KCNQ1 promoter, was inversely correlated with KCNQ1 expression in these patients (SI Appendix, Fig. S11). High TCF4 expression was significantly correlated with low patient survival probability (Fig. 7C). Although KCNQ1 was correlated with patient survival, we found no significant correlation between KCNQ1 expression and tumor stage (SI Appendix, Table S1).

Discussion

Our study demonstrates that KCNQ1 is a target gene for the Wnt/β-catenin pathway. We also uncovered a function of KCNQ1 in regulating β-catenin activity at the plasma membrane. The loss of KCNQ1 promotes the disruption of cell–cell contact, contributing to EMT, cell proliferation, and invasion. The function of KCNQ1 as an ion channel appears to be involved in these processes. We describe the molecular mechanisms of KCNQ1:β-catenin bidirectional interactions and a signaling pathway for the tumor suppressor activity of KCNQ1 in CRC. A number of studies have linked K+ channel deregulation to carcinogenesis (4, 5), but the underlying molecular mechanisms have remained largely unknown. In this study, we demonstrate a positive correlation between high KCNQ1 expression and CRC cell epithelial phenotype and patient survival in primary stage
CRC. The characterization of signaling pathways regulating ion channel expression in cancers is an open question, and very few cases, including the overexpression of the K+ channel oncogene EAG1 (17), have been documented so far. Here we demonstrate that differential KCNQ1 expression in CRC cell lines of varying cases, including the overexpression of the K+ channel expression in cancers is an open question, and very few CRC. The characterization of signaling pathways regulating ion expression had significantly better survival rates than those with low KCNQ1 and KCNE3. (C) Low TCF4 expression significantly correlated with longer relapse-free survival (P = 0.044).

Fig. 7. Correlation of KCNQ1:KCNE3 and TCF4 expression with CRC patient survival. Kaplan-Meier analysis of relapse-free survival from 286 colon cancer patients. KCNQ1 (A) and KCNE3 (B) expression significantly correlated with relapse-free survival (P = 0.022; P = 0.034). Patients with high KCNQ1 and KCNE3 expression had significantly better survival rates than those with low KCNQ1 and KCNE3. (C) Low TCF4 expression significantly correlated with longer relapse-free survival (P = 0.044).

Materials and Methods

CRC Cell Culture and Spheroid Formation. HEK293T and CHO cell lines, as well as the CRC cell lines HT29, HT29cl.19A, SW480, SW620, DLD-1, and HCT116, were maintained in Dulbecco’s modified Eagle’s or RPMI medium with 10% FBS. 3D-culture CRC spheroids were developed in Growth Factor Reduced Matrigel (BD Biosciences), as further described in SI Appendix, Materials and Methods. No human material requiring consent was used in our experiments.

Animals and Isolation of Colonic Crypts. Male Sprague-Dawley rats (200–250 g) were obtained from Janvier Labs and maintained at the laboratory animal house during a short period of 24 h (local ethics committee approval was
received from the University of Nice Sophia-Antipolis). Animals were killed by lethal intraperitoneal injection of pentobarbital. The colon was removed by dissection, and colonic crypts were isolated as previously described in ref. 12.

Plasmid Transfection and ShRNA Transduction. Two stable KCNQ1 shRNA knock-down cell lines were engineered using two different DNA sequences (SI Appendix, Materials and Methods) The double-stranded DNA sequences were inserted in the mammalian expression vector pSuperR Puro, a derivative of the pPIRE series. Transduction experiments were performed as previously described (28, 29). Cells were transiently transfected using lipofectamine 2000 (Invitrogen), according to the manufacturer’s recommendation. DLD-1 and SW480 cells were transfected with 2 μg hAN-TCF4 plasmid (30). HT29cl.19A and HT29 were transfected in suspension with 2 μg β-catenin7373 plasmid (31). KCNQ1 transduction of HTC116 cells was performed as described previously (32). More details are given in SI Appendix, Materials and Methods.

Transepithelial Electrical Resistance. HT29cl.19A cells were grown on semi-permeable supports (Merck Millipore), and TER was measured in triplicate with an EVOM electrometer (WPI), using triplicates for each measurement every 2 for 10 d. More details are given in SI Appendix, Materials and Methods.

Immunoblotting and Immunofluorescence. Whole-cell lysates were prepared and subjected to 8% SDS/PAGE before proteins being transferred onto a nitrocellulose membrane. Membranes were probed with the primary antibody overnight at 4 °C. Reactive proteins were developed with HRP-conjugated secondary antibody and visualized with chemiluminescence reagent. Western blotting was performed as described previously (12). Bound antibodies were detected using rabbit anti IgG Alexa 488 (Invitrogen). Image acquisition was performed using a Zeiss LSM710 laser scanning confocal microscope.

Chromatin Immunoprecipitation. ChIP was carried out using a ChiP-IT High Sensitivity Kit (Active Motif), as previously described (33). More details are provided in SI Appendix, Materials and Methods.

In situ Proximity Ligation Assay. The DuoLink PLA (Olink Bioscience) was used to detect interactions among KCNQ1, β-catenin, and E-cadherin. HT29cl.19A cells were seeded on poly-L-lysine (40 μg/mL)-coated microscope slides. CRC cells and isolated rat colonic crypts were fixed with paraformaldehyde 4% and permeabilized with triton x100 (0.05%). Cells were immunolabeled with primary antibodies anti KCNQ1, Kv.1.1 (1:100) and anti β-catenin (1:100), or anti E-cadherin (1:100) for 1 h at 37 °C. The secondary antibodies with attached PLA probes were supplied in the DuoLink kit. Cellular PLA images were captured using an inverted Zeiss Axios Observer Z1 microscope (Zeiss). PLA dots per cells were quantified using Image J software.

 Luciferase Reporter Assay. CHO cells were plated at 2 × 10^4 cells/well in 96-well plates. One day after plating, the cells were washed once with PBS and transfected with either LightSwitch GoClones KCNQ1 reporter (Active Motif), LightSwitch GAPDH Promoter Control (positive control; Active Motif), or LightSwitch Random Promoter Control 1 (negative control; Active Motif), using FuGENE HD transfection reagent (Promega Corporation). After 24 h, transfected cells were treated with 40 nM GSK3-IX (Merck Millipore) for another 24 h. Luciferase activities were measured using the LightSwitch Luciferase Assay Kit and LightSwitch Assay Substrate optimized for use with RenSP luciferase, using one-step reagent addition (Active Motif).

Statistical Analysis. Mann–Whitney or Kruskal-Wallis nonparametric test were applied to compare significance between two or more groups, as appropriate. The frequency of lumen formation in HT29cl.19A and HCT116 spheroids was analyzed using Fisher’s exact test. Values of n number of experiments are given as mean ± SEM. A P value <0.05 was considered statistically significant.

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