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Article

Dysregulated Expression of Ion Channels in Sensory Neurons Enhances Action Potential Afterhyperpolarization in Patients with Chronic Kidney Disease–Associated Pruritus

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Abstract: Expression levels of Cav3.2, BKCa, and anoctamin 1 were previously found to be significantly elevated in patients with chronic kidney disease–associated pruritus (CKD-aP). On the other hand, the expression of TRPV1 was significantly reduced. We further compared CKD patients with and without CKD-aP in terms of the expression levels of several ion channels in the skin, including in peripheral nerve endings。Based on CKD-aP severity, subjects were divided into two groups: non-CKD-aP (no or slight pruritus; n = 16) and CKD-aP (mild, moderate, or severe pruritus; n = 16). Skin samples were obtained from the forearm or elbow during arteriovenous fistula surgery. We used quantitative real-time polymerase chain reaction to measure the skin expression levels of the following ion channels in the skin: Nav1.7, Kv7.2, TREK1, HCN2, TrkA, and Piezo2. RT-PCR analyses showed that CKD-aP patients had significantly higher levels of TREK1 and Piezo2 transcripts and significantly lower levels of HCN2 transcripts than non–CKD-aP patients. No significant differences were noted between groups in the expression of Nav1.7 or TrkA. Moreover, Kv7.2 transcripts were not detected in either group. In skin samples collected from CKD-aP patients, ion channel expression patterns were altered to enhance hyperpolarization of pruriceptive neurons.

Keywords: CKD-aP; ion channels; action potential; hyperpolarization; RT-PCR

1. Introduction

Approximately 40% of patients on dialysis suffer from chronic kidney disease–associated pruritus (CKD-aP), despite increasing availability of new antipruritic drugs, advanced hemodialysis techniques such as on-line hemodiafiltration, and new dialysis fluid purification methods [1]. The sensation of itching can be evoked by various kinds of exogenous and endogenous pruritogenic agents. When they bind to and activate relevant receptors expressed on free sensory nerve endings, the resulting neuronal signals are transmitted to the central nervous system. The cerebral cortex integrates multisensory information and interprets the signals as itching, thereby activating a scratching response.

Previously, we investigated skin samples collected from CKD-aP and non–CKD-aP patients, and immunohistochemically compared the expression levels of multiple pruritogenic substances such as histamine, acetylcholine, β-endorphin, endothelin 1, and homocysteine. All investigated pruritogens were detected in both patient groups, with no significant between-group differences [2]. In a study by Yamamoto et al. that investigated the relationship between CKD-aP severity and blood uremic toxins, no significant association was observed between the 5D-itch scale score and the levels of indoxyl sulfate, *p*-cresyl sulfate, and other blood uremic toxins [3]. Taking note of these findings, we hypothesized that the mechanisms of pruritus perception are more strongly associated with

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patterns of terminal receptor expression than differences in pruritogenic stimuli. This hypothesis was also supported by multiple studies suggesting the significant role of receptor dysregulation in the mechanisms of pain and pruritis [4,5].

The human GPCR family comprises approximately 800 members. When a pruritogenic agent binds to a GPCR molecule, it triggers a downstream cascade of stimulatory and inhibitory intracellular signaling pathways that involve the trimeric G protein, PKA, PLC, and calcium ion. These signals are integrated to modulate the functions of the ICs that regulate the generator potential [6]. Because the vast number of GPCR molecules did not allow us to select a promising subset suitable for our research, we focused attention on their downstream ICs.

In our previous study using quantitative real-time polymerase chain reaction (RT-PCR), we examined the patterns of IC expression in skin samples that contained sensory neurons, keratinocytes, mast cells, Langerhans cells, sebocytes, and hair follicle cells [106]. We showed that CKD-aP patients had significantly higher levels of Cav3.2, BKCa, and anoctamin-1 transcripts and significantly lower levels of transient receptor potential channel subtype vanilloid 1 (TRPV1) than non-CKD-aP patients. In the current study we extended our research by comparing the expression of several ion channels in the skin, including in peripheral nerve endings, between CKD patients with and without CKD-aP.

2. Results

2.1. Baseline Characteristics

No differences were observed between the two groups in term of age, gender, primary disease, duration of renal replacement therapy, presence of hepatitis B or C, number of patients receiving pruritus treatment, or the levels of corrected Ca, inorganic phosphorus, blood serum albumin, blood serum hsCRP, and blood serum ferritin (Table 2).

Table 2. Patient characteristics.

	Non-pruritus (n = 16)	Pruritus (n = 16)	*P* value
Degree of pruritus	none, slight	mild, moderate, severe	
Gender (F/M)	5/11	4/12	>0.05
Age (years)	69 ± 13	69 ± 13	>0.05
Original disease (DM/CGN/HT/unknown)	8/3/3/2	10/2/0/4	>0.05
HBV/HCV (n)	0/2	0/1	>0.05
Duration of HD (days)	7 (0–6729)	3 (0–2276)	>0.05
Albumin (d/dL)	3.2 ± 0.5	3.3 ± 0.6	>0.05
Corrected Ca (mg/dL)	9.0 ± 1.1	8.4 ± 1.6	>0.05
iP (mg/dL)	5.6 ± 2.0	5.4 ± 1.4	>0.05
i-PTH (pg/mL)	226 ± 182	293 ± 220	>0.05
hsCRP (mg/dL)	0.28 (0.01–1.35)	0.07 (0.02–2.12)	>0.05
Ferritin (ng/ml)	211 ± 165	171 ± 131	>0.05
Anti-pruritic therapy (nalfurafine, urea, predonisolone, crotamiton, diphenhydramine)	2 (13 %)	5 (31%)	>0.05

Values are presented as means ± SD, median (range), or numbers (percentages). *P*-values were calculated using the unpaired t-test, Mann-Whitney U-test, or chi-square test. DM, diabetes mellitus; CGN, chronic glomeluronephritis; HT, hypertension; hsCRP, hypersensitivity C-reactive protein.

2.2. RT-PCR Analysis of Ion Channels in the Skin

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The RT-PCR analysis in the present study showed that CKD-aP patients had significantly higher expression levels of TREK1 and Piezo2 transcripts and significantly lower expression levels of HCN2 than non–CKD-aP patients. Moreover, the expression levels of Nav1.7 and TrkA were not significantly different between groups, and no Kv7.2 transcripts were detected in either group (Table 3).

Table 3. Relative expression levels.

	Gene	Non-pruritus	Pruritus	*P* value
Nav1.7	SCN9A	1.04 ± 0.30 (n = 16)	1.01 ± 0.27 (n = 16)	0.741
Kv7.2	KCNQ2	No date (n = 16)	No date (n = 16)	
K2p2.1 (TREK1)	KCNK2	1.07 ± 0.37 (n = 16)	1.78 ± 0.73 (n = 16)	0.002
HCN2		1.06 ± 0.36 (n = 16)	0.78 ± 0.31 (n = 16)	0.025
TrkA	NTRK1	1.09 ± 0.46 (n = 16)	2.23 ± 0.68 (n = 16)	0.195
Piezo2		1.01 ± 0.45 (n = 16)	1.09 ± 0.46 (n = 16)	<0.001

Values are expressed as means ± SD. *P*-values were calculated using the unpaired *t*-test.

3. Discussion

There is known to be overlap between the receptors responsible for pruritus and pain [9-11]. ICs that regulate the action potentials (APs) of nociceptive peripheral nerves include the following: Na1.6, Nav1.7, Nav1.8, Nav1.9, Kv1.4, Kv3.4, Kv7.2, KCa, TREK1, TRAAK, Cav2.2, Cav3.2, TREM16A, TRPV1, TRPA1, HCN2, TrkA, and Piezo2 [12]. Of these, Nav1.7, Kv7.2, TREK1, HCN2, TrkA, and Piezo2 were chosen for analysis. The reasons for their selection and a discussion of relevant findings are discussed below.

At least six voltage-gated sodium channel subtypes (Nav1.1, Nav1.3, Nav1.6, Nav1.7, Nav1.8, and Nav1.9) are upregulated in sensory neurons. In particular, nav1.7 is predominantly expressed in peripheral sensory neurons. Nav1.7 is responsible for the rising phase of APs and plays a key role in setting the threshold for AP generation in primary sensory neurons [13]. Nav1.7 is important for pain and pruritus sensations in rodents and humans [14-16]. Although a previous rat model study suggested that increased local expression of Nav1.8 was associated with the pathogenesis of neuropathic pain [17], we found no significant difference in Nav1.7 expression between CKD-aP and non–CKD-aP patients. Our results suggested that Nav1.7 had negligible impact on pruritus sensation. Despite the fact Nav1.7 is a key IC that contributes to the steep AP upstroke in pruriceptive neurons, our results did not support the hypothesis that differences in Nav1.7 expression were involved in CKD-aP pathogenesis. Our results warrant further study to examine the relationship between other Nav subtypes (e.g., Nav1.6 and Nav1.9) and CKD-aP.

Potassium channels control neuronal excitability by influencing the duration, frequency, and amplitude of APs [18]. K2P channels give rise to leak (also called background) K^+ currents, causing resting potassium conductance and preventing excessive neuronal activation [19,20]. K2P channels are expressed in the central and somatic peripheral nervous systems, as well as in a number of non-neuronal mammalian tissues and organs [19]. TREK-1 (K2P 2.1) belongs to a novel family of mammalian K2P channels and is highly expressed in peripheral dorsal root ganglion neurons. The main role of TREK-1 is to control cell excitability and maintain the membrane potential below the depolarization threshold [21,22]. Our study showed that the cutaneous TREK1 expression level was

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significantly elevated in CKD-aP patients compared with non–CKD-aP patients. Elevated TREK1 expression suggests the suppression of neural excitability, depolarization, and neuronal firing. In our previous study [23], we found that BKCa, a known mediator of hyperpolarization, was expressed at significantly higher levels in CKD-aP patients than in non–CKD-aP patients. Similar to the role of BKCa in promoting hyperpolarization, increased expression levels of TREK1 were found to contribute to hyperpolarization of pruriceptive terminals in CKD-aP patients.

M-type (Kv7, KCNQ) potassium channels control the resting membrane potential of many neurons, including peripheral nociceptive sensory neurons [24]. M-channels are voltage-gated potassium channels that are formed by KCNQ2, KCNQ3, and KCNQ5. Neuronal KCNQ channels (KCNQ2, 3, 5) exhibit the M-current (I_M), a slowly activating, non-inactivating outward rectifying potassium current that can be inhibited by muscarinic stimulation [25]. Notably, KCNQ2 has been identified in the somatosensory system [25]. The potassium channel subunits Kv7.2 and Kv7.3 play a key role in stabilizing neuronal activity [26]. King et al. reported that the loss of Kv7.2 activity increased the excitability of primary sensory neurons [27]. However, Kv7.2 transcripts were not detected in the skin samples of CKD-aP and non–CKD-aP patients, suggesting that Kv7.2 does not play a major role in the pathogenesis of pruritus.

HCN channels are permeable to both K^+ and Na^+ and are responsible for voltage-gated inward rectifying (I_h) currents that are activated during hyperpolarization [28,29]. HCN channels underlie the depolarization modulating the rhythmic generation of APs, and they also contribute to the resting membrane potential and modify the waveforms of propagated synaptic and generator potentials in peripheral sensory neurons that mediate pain sensation [28,30]. The HCN ion channel family comprises 4 isoforms (HCN1 to HCN4). HCN2 modulates the AP firing rate in nociceptive neurons and plays a critical role in all modes of inflammatory and neuropathic pain [29,31,32]. The binding of cAMP to HCN2 channels shifts the activation curve in the positive direction as a function of membrane voltage, and thereby increases the I_h current [31,32]. Our results showed that skin HCN2 expression was significantly lower in CKD-aP patients than in non–CKD-aP patients. Lower expression levels of HCN2 suggested a reduced I_h current amplitude, which could possibly delay the return to the resting potential from the undershoot during the refractory period.

Nerve growth factor (NGF) is a well-known neurotrophic factor that also acts as a mediator of pain, pruritus, and inflammation [33]. The NTRK1 gene encodes TrkA, a receptor tyrosine kinase for NGF (NGF–TrkA system) [34,35]. Human skin, including nerves and sensory corpuscles, displays immunoreactivity to low-(p75) and high-affinity (TrkA-like) NGF receptors [36]. Our results showed no significant differences in TrkA transcript expression between CKD-aP and non–CKD-aP patients, suggesting that the NGF–TrkA system did not have a major impact on CKD-aP pathogenesis.

Piezo is a mechanosensitive cation channel responsible for stretch-mediated Ca^{2+} and Na^+ influx in multiple types of cells. Piezo1 mediates shear stress and stretch-induced transmembrane currents, mainly in nonneuronal cells, whereas Piezo2 is predominantly expressed in primary sensory neurons, where it mediates proprioception, touch perception, and detection of noxious mechanical stimuli [7]. In disease states (e.g., aging or dry skin), innocuous mechanical stimuli can provoke pathologic sensations such as allokinesis (mechanical itch), a phenomenon modulated at least partly by Piezo2 [7,37]. Feng et al. showed that cutaneous Piezo2 channel–Merkel cell signaling is critical in modulating the conversion of touch to itch [37]. The current study showed that CKD-aP patients had significantly elevated expression levels of skin Piezo2 than non–CKD-aP patients, suggesting that CKD-aP patients were more likely to develop allokinesis.

Overall, the RT-PCR analyses of skin ion channels reported here showed that CKD-aP patients had significantly higher TREK1 and Piezo2 expression levels and significantly lower HCN2 levels than non–CKD-aP patients. Moreover, no significant differences were noted in Nav1.7 or TrkA expression levels, and Kv7.2 transcripts were not detected in

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either group. In combination with our previous findings [23], these results indicate that CKD-aP skin samples had significantly higher TREK1, Piezo2, Cav3.2, BKCa, and anoctamin-1 expression levels and significantly lower HCN2 and TRPV1 expression levels than non–CKD-aP skin samples. Moreover, no significant between-group differences were observed in Nav1.7, TrkA, Cav2.2, or ASIC expression levels, and Nav1.8, Kv1.4, Kv7.2, and TRPA1 transcripts could not be detected in either group.

Assuming that IC expression levels in peripheral nerves correlate with those in other skin cells, our results suggest that elevated levels of TREK1, BKCa, and anoctamin 1 increase the afterhyperpolarization amplitude, defined as the absolute difference between the resting potential and the minimum membrane potential attained during repolarization. In addition, lower HCN2 expression levels observed in CKD-aP patients suggested that compared with non–CKD-aP patients, these patients had a smaller I_h current amplitude and the membrane potential required a longer time to return to the resting state during the hyperpolarization period. We previously reported that the skin expression levels of the calcium-activated chloride channel anoctamin 1 were significantly upregulated in CKD-aP patients [23]. Unlike the neural cells in dorsal root ganglion, the neural cells at peripheral nerve endings are considered to be mature neurons, so the concentration of intracellular chlorine seem to be low. Given that mature neuronal and other excitable cells generally have low intracellular chloride concentrations [38], the activation of anoctamin 1 may enhance hyperpolarization of the peripheral sensory nerves. While much remains to be discovered about the role of TRPV1 in shaping the action potential waveform, TRPV1 is likely to contribute to depolarization because it facilitates transmembrane sodium and calcium ion influx. Consequently, our finding that TRPV1 was downregulated in CKD-aP patients suggested that APs had a longer rising phase in these patients. Overall, this study suggested that compared with controls, CKD-aP patients had a longer AP cycle due to upregulation of ICs that induced a greater afterhyperpolarization amplitude (Figure 1).

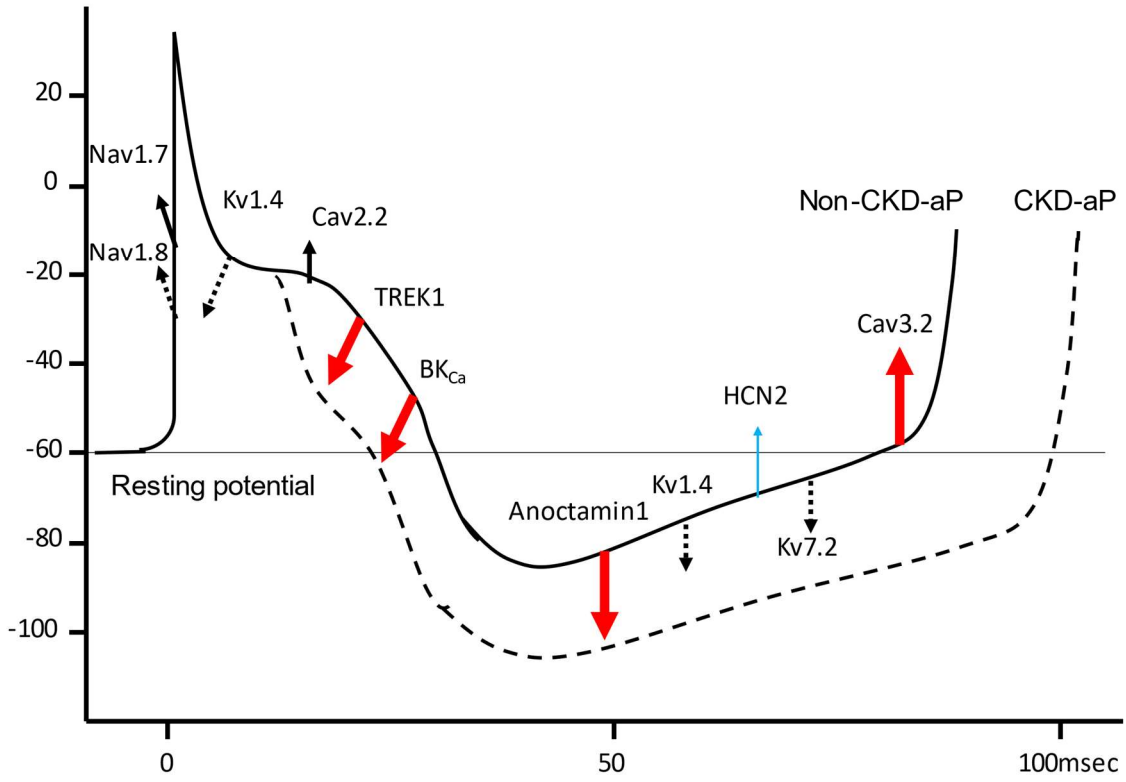

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Figure 1. Action potential is shaped by the combination of Ion channels. Nav1.7-Voltage-gated sodium channel 1.7, Nav1.8-Voltage-gated sodium channel 1.8, Kv1.4-Voltage-gated potassium channel 1.4, BK_{Ca}-Ca^{2+}-activated K^+ channel (big conductance), Cav2.2- Voltage-gated calcium channel 2.2, Cav3.2- Voltage-gated calcium channel 3.2, HCN2-Hyperpolarization-activated, cyclic-nucleotide-gated channel 2.

→ The expression of ion channel in the skin was significantly elevated in patients with CKD-aP than those without CKD-aP.

→ The expression of ion channel was significantly reduced in patients with CKD-aP compared with those without CKD-aP.

→ There was no significant difference in the expression of ion channel between patients with CKD-aP and those without CKD-aP.

▪▪▪▪▪▶ The expression of ion channel was not detected.

Many studies reported that chronic pain downregulated Kv1.4 and BKCa and upregulated other peripheral nociceptive nerve ICs [39-43]. Moreover, a study of a chronic pain model showed that BKCa blockade suppressed afterhyperpolarization [44], and a separate study showed that pruritus-related pathways were associated with a lower AP frequency than pain pathways [45]. In light of these findings, the results of the present study suggest that pruritus and pain pathways are electrophysiologically mediated by ICs that promote hyperpolarization and depolarization, respectively. A variety of receptors involved in AP generation help discriminate between pain and pruritus sensations, possibly depending on their spatial locations, distribution patterns, morphology, and other properties. Our findings suggest that temporal discharge patterns of APs are key components underlying pruritus, thereby supporting the spatial contrast theory [46]. Differential expression of ICs is likely to contribute functional diversity to sensory neuron signaling.

Cav3.2 may play a key role in AP generation in CKD-aP. The activity of Cav3.2 depends not only on the membrane potential but also on various neurotransmitters and intracellular second messengers. Zinc ions and hydrogen sulfide facilitate pruritus processing by enhancing Cav3.2 activity [39]. We previously reported that Cav3.2 was overexpressed in CKD-aP patients [23]. The overexpression of Cav3.2 activates voltage-gated sodium channels by elevating the membrane potential, which in turn increases the frequency of AP discharge. The potentially key role of Cav3.2 in CKD-aP may be further supported by the finding that the expression of Cav3.2 on peripheral nerve terminals is upregulated in chronic pain [39]. TRPV1 may also play a major role in AP generation because it can activate voltage-gated sodium channels. However, given our previous finding that TRPV1 was downregulated in CKD-aP patients, this possibility is unlikely.

The mammalian olfactory system possesses receptors that discriminate various odorant molecules. It employs a combinatorial receptor coding scheme to differentiate odor identities [47]. Various combinations of olfactory receptor activation produce different generator potential patterns, which are rectified to generate APs that are transmitted to the central nervous system. Similarly, pruritus sensations are often perceived and described in a variety of ways. Typical sensory descriptors of pruritus include crawling (formication), prickling, wriggling, tickling, tingling, stinging, burning, and pinching. These distinct perceptions may be attributable to different activated combinations of peripheral sensory nerve ICs, which deliver different afferent signals to the central nervous system. Certain descriptors of pruritus, such as crawling and tickling, are apparently related to tactile sensations, suggesting the involvement of Piezo2.

Our series of RT-PCR analyses using beta-2 microglobulin as an internal control did not detect transcripts encoding Nav1.8, Kv1.4, Kv7.2, or TRPA1. Because of their relative nature, our results may allow for multiple interpretations. One is that the functionalities of Nav1.8, Kv1.4, and Kv7.2 are of greater importance in pruritus pathogenesis than their expression levels, similar to the case for the nonselective cationic IC TRPA1 [48].

Regarding ICs whose expressions were confined to cutaneous sensory nerve endings, TREK1 and Cav3.2 were significantly upregulated and HCN2 was significantly downregulated in CKD-aP patients compared with non–CKD-aP patients. No significant between-

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group differences were noted for Nav1.7, TrkA, Cav2.2, or ASIC. Overall, our results suggest that new therapies for pruritus might include TREK1 or Piezo2 antagonists, HCN2 agonists, and their combinations. Moreover, our findings indicate the potential utility of developing new agonists and antagonists that either suppress hyperpolarization-inducing ICs or activate depolarization-inducing ICs.

Our results warrant further investigation of Toll-like receptors and similar ICs. Additional research should also focus on the intracellular signaling pathways upstream of GPCRs.

This study had several limitations. First, the sample size was small. Second, we did not isolate peripheral nerves innervating human skin, which would have enabled us to focus on ICs expressed on peripheral nerve terminals. To address this issue, double immunofluorescence staining of ICs and use of a neuronal marker (e.g., protein gene product 9.5) may be beneficial. Third, we did not perform Western blotting, in situ hybridization, or immunocytochemistry analyses, and also did not investigate differences in IC function between CKD-aP and non–CKD-aP patients, underscoring the need for electrophysiological IC research.

4. Conclusions

ICs that enhance hyperpolarization were upregulated in sensory nerve terminals innervating the skin of CKD-aP patients.

5. Materials and Methods

5.1. Subjects

This cross-sectional study was approved by the Ethics Committee of Jusendo General Hospital. (Approval code: No.126). Each patient gave written informed consent. Between February 2016 and December 2020, we performed arteriovenous fistula surgery in 113 patients with stage 5 CKD. Thirty-two patients (nine women and 23 men, 69 ± 13 years old) agreed to participate in the study.

Patients with concomitant dermatitis, e.g., atopic dermatitis or psoriasis, were excluded. If pruritus severity differed between day and night, the higher score (i.e., more severe pruritus) was defined as the pruritus score. Subjects were divided into two groups: non-CKD-aP (no or slight pruritus; n = 16) and CKD-aP (mild, moderate, or severe pruritus; n = 16) (Table1).

Table 1. Definitions of Shiratori's itch severity scores.

Score (severity)	Daytime symptoms	Nighttime symptoms
4 (severe)	Intolerable itching, worsened instead of relieved by scratching. Cannot focus on work or study.	Can hardly sleep because of itching. Scratching all the time, but itching intensifies with scratching.
3 (moderate)	Scratching even in the presence of others. Irritation as a result of itching and continuous scratching.	Wake up because of itching. Can fall asleep again after scratching, but continue to scratch unconsciously while sleeping.
2 (mild)	Itch sensation is relieved by light, occasional scratching. Not too disturbing.	Moderate itchiness is relieved by scratching. No awakening due to itching.
1 (slight)	Feel itchy sometimes, but tolerable without scratching	Feel slightly itchy when going to sleep, but do not need to scratch. Sleep well.
0 (no symptoms)	Hardly feel itchy or do not feel itchy at all.	Hardly feel itchy or do not feel itchy at all.

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5.2. Study Design

We compared the two groups in terms of age, gender, primary disease, presence of hepatitis B or C, and number of patients receiving treatment for pruritus, as well as levels of blood serum albumin, corrected Ca, inorganic phosphorus, intact parathyroid hormone (intact-PTH), blood serum hypersensitive C-reactive protein (hs-CRP), and blood ferritin. Skin samples, each with an area of about 10 × 5 mm^2, were obtained from the forearm or elbow immediately after starting the arteriovenous fistula surgery.

5.3. Quantitative RT-PCR

Ion channels constitutively expressed in cutaneous peripheral nerve endings in healthy individuals include the following: voltage-gated sodium channel subtypes Nav1.6, Nav1.7 (SCN9A), Nav1.8, and Nav1.9; voltage-gated potassium channel subtypes Kv1.4, Kv3.4, and Kv7.2 (KCNQ2); two-pore-domain potassium (K2P) channel subtypes TREK1 (KCNK2) and TRAAK; N-type and T-type voltage-gated calcium channel subtypes (Cav2.2 and Cav3.2, respectively); TRPV1 and transient receptor potential channel subtype ankyrin 1 (TRPA1); and calcium-activated potassium channel (KCa), big-conductance calcium-activated potassium channel (BKCa), calcium-activated chloride channel anoctsamin-1 (TMEM16A), acid-sensing ion channel type 1 (ASIC1), tropomyosin receptor kinase A (TrkA/NTRK1), and hyperpolarization-activated cyclic nucleotide-gated (HCN) channel subtype HCN2. Among the ICs constitutively expressed in skin tissue containing peripheral nerve endings, we measured the expression levels of Nav1.7, Kv7.2, TREK1, HCN2, TrkA, and Piezo-type mechanosensitive ion channel component 2 (Piezo2). While Nav1.7, Kv7.2, and TREK1 is expressed only in cutaneous peripheral nerve endings, HCN2, TrkA, and Piezo2 are also expressed on non-neuronal cells, such as keratinocytes, fibroblasts, smooth muscle cells, vascular endothelial cells, Merkel cells, Langerhans cells, lymphocytes, and macrophages [7].

Gene expression levels of ion channels were determined after reverse transcription of RNA samples by quantitative PCR using an ABI PRISM 7000 Sequence Detector (Thermo Fisher Scientific, Waltham, MA, USA), as described previously [8]. Pre-made TaqMan® Gene Expression Assays for humans were used. Total RNA was isolated from biopsied skin specimens using TRIzol (Thermo Fisher Scientific). cDNA was synthesized from total RNA by RT-PCR using SuperScript™ VILO™ Naster Mix (Thermo Fisher Scientific) following the manufacture's protocol. cDNA was synthesized from 1 μg of total RNA. To standardize quantitation, beta-2 microglobulin was amplified simultaneously. The expression level of each gene is presented as the fold increase in the CKD-aP group compared to the non–CKD-aP group.

5.4. Statistical Analysis

All values are expressed as means ± standard deviations (SD) for normally distributed data, medians (ranges) for non-normally distributed data, and numbers (percentages). No data points were excluded. All statistical analyses were performed using the unpaired t-test or m × n chi-square test for categorical outcomes. P-values <0.05 were considered to be statistically significant.

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