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SCN9A Mutations Define Primary Erythermalgia as a Neuropathic Disorder of Voltage Gated Sodium Channels

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Primary erythermalgia is a rare disorder characterized by recurrent attacks of red, warm and painful hands, and/or feet. We previously localized the gene for primary erythermalgia to a 7.94 cM region on chromosome 2q. Recently, Yang et al identified two missense mutations of the sodium channel α subunit SCN9A in patients with erythermalgia. The presence of voltage-gated sodium channels in sensory neurons is thought to play a crucial role in several chronic painful neuropathies. We examined four different families and two sporadic cases and detected missense sequence variants in SCN9A to be present in primary erythermalgia patients. A total of five of six mutations were located in highly conserved regions. One family with autosomal dominantly inherited erythermalgia was double heterozygous for two separate SCN9A mutations. These data establish primary erythermalgia as a neuropathic disorder and offers hope for treatment of this incapacitating painful disorder.

Key words: autosomal dominant/cloning/erythromelalgia/heterozygous

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Primary erythermalgia is an autosomal dominant condition that is featured by intermittent attacks of red, warm, swollen extremities (Drenth and Michiels, 1990). Patients develop complaints at young age, mostly within the first decade of life. Typically, attacks are provoked by warmth and exercise, but in more advanced cases, symptoms can progress to multiple flares a day. With progression symptoms can extend to upper arms and legs and even torso and can be present constantly (Drenth and Michiels, 1994). The pathology of primary erythermalgia is unknown although recent studies have suggested that increased microvascular arteriovenous shunting in deep dermal plexa inducing hypoxia is responsible for the pain (Mork et al, 2000). It is not known however, whether this actually underlies primary erythermalgia or that it arises as a secondary phenomenon. On the other hand there are indications for an impaired small fiber function in primary erythermalgia suggesting a neuropathic component (Orstavik et al, 2004). Treatment of primary erythermalgia is considered to be very difficult, and although a great variety of therapeutic options have been proposed, none of them have brought uniform and sustained relief. Primary erythermalgia can be regarded as a human model for some types of pain, and as such it can offer great insight in the pathophysiology of pain (Orstavik et al, 2003). The familial character of primary erythermalgia suggests that gene mutations underlie the disease. Identification of the gene and study of the

incriminated protein can teach us much about the pathophysiology of primary erythralgia and also offer us an opening to search for treatment.

A recent genome-wide linkage study with five kindreds with erythralgia placed the gene for primary erythralgia on chromosome 2q (Drenth et al, 2001). An analysis of recombinant events in one family identified marker D2S2370 at the centromeric end and marker D2S1776 at the telomeric end as flanking markers. Inspection of the linked interval reveals that there is a cluster of five sodium channels genes (SCN1a, SCN2a, SCN3a, SCN7a, SCN9A) located on chromosome 2q. Voltage-sensitive sodium channels play a critical role in rapid membrane depolarization that is responsible for the rising phase of the action potential in most excitable cells (Catterall, 2000). The voltage sensitive sodium channel complex is composed of a subunits of 260 kDa that forms the voltage sensitive and ion-selective pore and smaller auxiliary b subunit(s) of 33–36 kDa, that can modulate the kinetics and voltage dependence of channel gating of the a subunits. The voltage-gated sodium channel subunits encoded by SCN1a, SCN2a, SCN3a, SCN7a on chromosome 2q are highly related, share at least 85% of the amino acid sequence for the transmembrane and extracellular domains, and are all tetrodotoxin sensitive (Chan et al, 2002). Three of the genes (SCN1a, SCN2a, SCN3a) encode for voltage-gated sodium channel subunits that are mainly expressed in the central nervous system but one (SCN9A), encoding for Nav1.7, is predominantly expressed in the peripheral nervous system (Klugbauer et al, 1995; Toledo-Aral et al, 1997).

Indeed, on the basis of available linkage data, Chinese researchers examined a three-generation family with inherited primary erythralgia and a isolated case and sequenced the sodium channel alpha subunit SCN9A in their patients (Kanadia et al, 2003). These investigators discovered a missense mutation (T2573A) that segregated with the disease in a family, whereas a T2543C mutation was present in their single sporadic case. This suggests that SCN9A mutations cause primary erythralgia, although confirmation is lacking to date.

Therefore, we hypothesized that mutations in SCN9A might be associated with painful neuropathy as is seen in primary erythralgia. To test the hypothesis that SCN9A mutations cause primary erythralgia, we sequenced this gene in patients stemming from four families and two sporadic cases.

Results

Mutational analysis We analyzed DNA from familial cases from a total of four families and two sporadic cases for SCN9A mutations by automated bidirectional sequencing of PCR-amplified genomic DNA for each of the 26 SCN9A exons including the exon–intron junctions. In four familial cases and a single sporadic case, we identified single-nuc-leotide substitutions that were spread over the SCN9A genome, all of which resulted in missense amino acid substitutions. Table I summarizes the various SCN9A mutations that were found among our patients. We detected a SCN9A mutation (T2543C) in a proband from French family (#2) (Fig 1). This mutation was previously described in a sporadic Chinese patient (Yang et al, 2004). We detected a novel C1185A mutation in a Dutch patient from family 3 with very severe primary erythralgia. In another French family (#4) we found a novel T647C mutation in the SCN9A sequence that was present in the affected father as well as in his affected daughter. We detected a C2572T mutation in a proband from a Canadian family (#5) with an especially severe phenotype, and this mutation changes the amino acid composition from leucine to phenylalanine (L858F). Interestingly, another mutation (T2573A) in the same codon (858) has been described in association with primary erythralgia in a Chinese family but in this case the mutation changes leucine for histidine. The proband from this family is double heterozygous at the same locus for a second mutation C1828A that changes the amino acid sequence at codon 610 from proline to threonine. We detected a missense mutation (C3448T) in exon 18 in one of our two sporadic primary erythralgia patients. In the last sporadic patient, bidirectional genomic sequencing failed to identify any SCN9A variants. The clinical features of this patient were comparable with other cases in whom we did detect mutations. The detected SCN9A sequence variants were not observed in any of the 100 control chromosomes and, when tested, segregated with the disease in families. Four mutations (N395K, F216S, I848T, and L858F) are located in the sodium channel pore region whereas two mutations (P610T and R1150W) are outside the ion transport region. Polymorphisms During our studies we detected five noncoding known SCN9A polymorphisms (A174G, G444A, T1119C, A1266G,

T1287A). These were mostly seen in a heterozygous state, but we also observed homozygous T1119C, A1266G, T1287A variants. Multiple alignment In order to assess the relative importance of the detected mutations we decided to perform multiple alignment of nucleotide sequence of SCN9A using the MULTALIN program. (<http://prodes.toulouse.inra.fr/multalin/multalin.html>) Sequences related to SCN9A were identified by BLAST searches (<http://www.ncbi.nlm.nih.gov/BLAST/>). These sequences included HS, Homo sapiens; CP, Cynops pyrrhogaster; RN, Rattus norvegicus; OC, Oryctolagus cuniculus; and MM, Mus musculus. Table II shows the three nucleotides that encode a codon in the human mRNA sequence. SCN9A mutations in primary erythermalgia affect nucleotides that are highly conserved among a wide range of species. This suggest that these mutations affect structurally and/or mechanistic important residues in the sequence. The C3448T mutation that was detected in a sporadic case is a notable exception. The triplet that encodes codon 1150 is variable and only O.cuniculus shares the exact same nucleotides with men. The fact that the mutation does not affect a highly conserved nucleotide sequence might suggest that this mutation does not cause the phenotype but that it is rather an innocent sequence variation.

Discussion

This report reports the identification of the SCN9A gene as responsible for primary erythermalgia on the basis of available positional cloning data. Our results confirm the earlier report that had detected SCN9A mutations to a single family and a sporadic case (Yang et al, 2004). Our conclusion is based on discovery of six missense mutations, none of which occurred in 4100 unaffected control chromosomes among a sample of for primary erythermalgia families and a sporadic case. All mutations alter the amino acid composition of the protein and all, but one, occur in highly conserved regions of the SCN9A sequence. On the other hand, the results from this study should be interpreted with caution. It is unsure whether the detected SCN9A sequence variants actually cause the disease. Although the genetic data may infer causality, specific functional data are lacking to date.

SCN9A is part of a cluster of sodium channels genes on chromosome 2 that probably arose by duplication of a common ancestral locus. SCN9A encodes the Nav1.7 sodium channel which is thought to play a crucial role in the establishment of the hyperexcitability state of the sensory neurons that contribute to the abnormal processing of nociceptive and/or tactile information. The presence of voltage-gated sodium channels in sensory neurons plays crucial role in several chronic painful neuropathies (Lai et al, 2003). For instance, in painful neuromas, a clinical situation that can arise after trauma to a peripheral nerve, there is evidence of Nav1.7 accumulation in histological sections (Kretschmer et al, 2002). Transfection experiments for two SCN9A mutations (I848T or L858H) associated with primary erythermalgia indicate that these mutations shifted the voltage dependence of activation in the hyperpolarizing direction (Cummins et al, 2004). Deactivation of the Nav1.7 channel was slowed by both mutations and the size of ramp currents produced by the sodium channels in response to slow depolarizations was likewise increased. This might suggest that excitability of sensory neurons that express the Nav1.7 channel is increased. These findings corroborate with recent neurophysiological experiments (Davis et al, 2003). In another study with erythermalgia patients, microneurographic recordings from single C-nociceptive fibers demonstrated reduced conduction velocities and increased activity-dependent slowing of the conduction velocity of afferent C-nociceptive fibers (Orstavik et al, 2003). In addition, mechano-insensitive fibers appeared to be spontaneously active or to be sensitized to mechanical stimuli. These mechano-insensitive C-nociceptors can contribute to the observed skin vasodilation by the release of vasoactive neuropeptides. As such, hyperexcitability of Cnociceptive fibers might be responsible for redness and warming as well as the pain in erythermalgia. It is possible that erythermalgia is caused by a gain-of-function of Nav1.7 channel. Nav1.7 channel knockout mice for example, have increased mechanical and thermal pain thresholds and reduced inflammatory pain response which emphasizes the crucial role of Nav1.7 in mediating pain (Nassar et al, 2004).

The identification of SCN9A mutations in primary erythermalgia represents a paradigm shift in the thinking about the pathogenesis of the disease. Former hypotheses were formulated on the basis that maldistribution of blood flow were the basis of the symptoms (Littleford et al, 1999). According to this concept, the blood flow over the arteriovenous shunts is increased, whereas nutritive perfusion is decreased. The inadequate nutritive blood flow induces superficial hypoxia, leading to an acidic pH and the

accumulation of vasodilatory mediators. When these vasodilatory mediators reach the arteriovenous shunts a vicious circle is initiated. Increased flow through these thermoregulatory shunts will further reduce nutritive perfusion and in addition, increase temperature and worsen the hypoxia because of increased energy consumption. The discovery of SCN9A mutations affecting Nav1.7 in these patients lends support to the concept that primary erythralgia is a small fiber neuropathic disorder (Davis et al, 2003).

The pathophysiology of a phenotypically closely related disorder, erythromelgia, sharply contrasts with that of primary erythralgia. In erythromelgia, patients likewise suffer from red and congested extremities with raised skin temperature and painful burning sensations. The basis for the disease is caused by the effects of thrombocythemia, which leads to thrombotic occlusions of arterioles and small arteries. This leads to platelet-mediated arteriolar inflammation and fibromuscular intimal proliferation. Aspirin induces normalization of the shortened platelet survival in thrombocythemia and reverses the inflammatory components of erythromelgia together with an improvement of the ischemic complications (Michiels et al, 1985). We have never found evidence for elevated platelet count in our patients, nor has aspirin provided clinical relief in any of our patients.

Our findings bring new hopes for the treatment of our patients. Neuropathic pain in primary erythralgia is considered to be very problematic condition in terms of analgesic therapy (Orstavik et al, 2004). In principle, voltage-gated sodium channels are targets for several drugs, such as local anesthetics (lidocaine), systemic antiarrhythmics (mexiletine), and antiepileptic drugs such as phenytoin. Indeed, in family 3 a single intravenous lidocaine infusion followed by oral mexiletine (600 mg daily) rapidly improved the symptoms (Legroux-Crespel et al, 2003). A similar approach was successful in patients with erythralgia (Kuhnert et al, 1999; Jang et al, 2004) which suggest that blocking of the voltage-gated sodium channels is a valid therapeutic option. Potentially, Nav1.7 may be a target for a channel-directed analgesic drug, and thus a specific treatment for primary erythralgia. Apart from its presence in peripheral neurons, Nav1.7 is being found in sympathetic neurons, and pharmacological channel blocking may, in principle, lead to undesired side effects. On the other hand, targeting Nav1.7 could influence nociceptive more than non-nociceptive neurons. In any case, our findings open the avenue for a well-directed search for novel Nav1.7 targeting drugs that potentially could benefit primary erythralgia patients.

Material and Methods

Subjects Blood samples for DNA analysis were obtained from subjects belonging to four families and two sporadic cases with primary erythralgia. The study was approved by the institutional review board at the University Medical Center St Radboud, Nijmegen, the Netherlands and written informed consent was obtained from the subjects. The study was conducted according to the Declaration of Helsinki Principles. All patients included in this study had been clinically evaluated by experienced physicians and the diagnosis was confirmed by the patient's history and clinical findings. All patients fulfilled the set of diagnostic criteria for primary erythralgia (Drenth and Michiels, 1994). Briefly these criteria were attacks of bilateral or symmetric burning pain in hands or feet, initiation or aggravation of attacks by standing, exercise, or exposure to heat, relief by elevation and cold, with attacks when the affected parts are warm, flushed, and congested (Fig 2) and lastly the disorder is largely refractory to treatment. Four families included in

this study have been subjects of previous studies (Fig 3) (Thompson et al, 1979; Martin et al, 1994; Guillet et al, 1995; Legroux- Crespel et al, 2003). Family 2 and 4 stem from France, family 3 comes from the Netherlands. The symptoms of the affected daughter of family 3 had progressed since the last description (Drenth et al, 2001) to such a degree that the symptoms were constantly present and that pain management up to administration of anesthetics via an epidural catheter was largely ineffective. She had large ulcers on lower leg and feet, probably as a result of prolonged cooling, and she developed sepsis and died as a result at the age of 39 y. Family 5 originates from Canada, whereas family 3 and 4 are three member families each with one affected parent (family 3 mother, family 4 father) and one affected daughter. The family history for the two sporadic cases was negative. DNA sequencing and mutation detection Genomic DNA was purified from leukocytes from fresh drawn blood or from Epstein– Barr immortalized cell lines according to established protocols. Primers were designed with the Oligo 4.0 program (Table III). Mutation screening was carried out for all 26 exons that constitute the SCN9A open reading frame. Genomic DNA was amplified by PCR with oligonucleotide primers complementary to flanking intronic sequences. The 50 mL reaction mixture contained 200 ng of genomic DNA, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% TRITON, 2mM MgCl₂, 0.25 mM dNTP, 100 ng of forward and reverse primers, and 3.0 U Taq-DNA-polymerase. The PCR conditions were 5 min at 95°C, then 35 cycles of 30 s at 95°C, 30 s at T_m of the used primer set, and 1 min at 72°C, and finally an elongation step at 72°C for 5 min. The PCR products were purified after electrophoresis on an agarose gel with the QIAEXII Gel Extraction Kit (Qiagen, Hilden, Germany) With the purified amplicons we performed sequencing using the BigDye terminator kit (Perkin Elmer Applied Biosystems, Boston, Massachusetts) according to the manufacturer's manual and the primers used in the PCR reaction. Sequences were analyzed on an ABI3700 capillary sequencer (Perkin Elmer Applied Biosystems). Sequence electropherograms were compared with gene sequence from GenBank and control samples. Further, we tested sequence variants for segregation among family members, if available.

We thank Deborah Mosarski and the members of The Erythromelalgia Association (TEA), for their valuable advice and dedication to our project. We are indebted to the study participants for their time and energy invested in this research effort. Saskia van de Velde Visser and Teun Baijens provided assistance with the cell cultures of Epstein–Barr transfected cell lines. Joost P. H. Drenth is a recipient of a ZON-MW VIDI research grant.

DOI: 10.1111/j.0022-202X.2005.23737.x

Manuscript received November 19, 2004; revised February 7, 2005; accepted for publication February 9, 2005

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