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Assessment of sodium channel mutations in Makah tribal members of the U.S. Pacific Northwest as a potential mechanism of resistance to paralytic shellfish poisoning

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ABSTRACT

The Makah Tribe of Neah Bay, Washington, has historically relied on the subsistence harvest of coastal seafood, including shellfish, which remains an important cultural and ceremonial resource. Tribal legend describes visitors from other tribes that died from eating shellfish collected on Makah lands. These deaths were believed to be caused by paralytic shellfish poisoning, a human illness caused by ingestion of shellfish contaminated with saxitoxins, which are produced by toxin-producing marine dinoflagellates on which the shellfish feed. These paralytic shellfish toxins include saxitoxin, a potent Na^+ channel antagonist that binds to the pore region of voltage gated Na^+ channels. Amino acid mutations in the Na^+ channel pore have been demonstrated to confer resistance to saxitoxin in softshell clam populations exposed to paralytic shellfish toxins present in their environment. Because of the notion of resistance to paralytic shellfish toxins, the study aimed to determine if a resistance strategy was possible in humans with historical exposure to toxins in shellfish. We collected, extracted and purified DNA from buccal swabs of 83 volunteer Makah tribal members and sequenced the skeletal muscle Na^+ channel (Nav1.4) at nine loci to characterize potential mutations in the relevant saxitoxin binding regions. No mutations of these specific regions were identified after comparison to a reference sequence. This study suggests that any resistance of Makah tribal members to saxitoxin, if present, is not a function of Nav1.4 modification, but may be due to mutations in neuronal or cardiac sodium channels, or some other mechanism unrelated to sodium channel function.

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1. Introduction

Human consumption of shellfish that feed on toxic algae can cause paralytic shellfish poisoning (PSP), a public health hazard that can cause severe economic losses globally due to bans on harvesting of contaminated shellfish. Paralytic shellfish toxin (PST)-producing dinoflagellates (e.g. *Alexandrium* spp.) cause toxic blooms ('red tides') in North America and worldwide. Records of PSP in the Pacific Northwest date back as early as June 15, 1793 (Vancouver, 1798), when a member of Captain George Vancouver's exploration team died from eating contaminated mussels in the uncharted coastline of what is now known as British Columbia. Additionally, 100 Russian hunters died from consuming toxic mussels in 1799 near Sitka, Alaska (Halstead, 1965).

The death of three people and illness of two others after their consumption of mussels and butter clams from the beach in Sekiu, WA in 1942 was the first evidence of high levels of PSTs in Washington State. Three members of the Ucluelet Tribe died after eating mussels containing PSTs on the west coast of Vancouver Island, British Columbia, Canada three days prior to the mortalities

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in Sekiu, suggesting that this event was probably widespread in the Pacific Northwest (Trainer et al., 2003). From 1942 to 1957, Washington State monitoring was sporadic, and temporarily stopped in 1946 due to blanket closures that were in effect at this time (Lilja, 1978), however these PSP outbreaks resulted in a mandatory monitoring program for PSTs that was implemented in Washington State in 1957. Annual shellfish harvest closures (except for razor clams) on the outer coast of Washington State continue in the present day and are enforced from Port Angeles on the Strait of Juan de Fuca, south to the mouth of the Columbia River (Fig. 1) for the period from April 1 to October 31 (Horner et al., 2011).

Paralytic shellfish toxins block conduction of the nerve impulse by interfering with the voltage-dependent increase in Na^+ ion conductance that generates the action potential (Hille, 1968; Narahashi and Moore, 1968), leading to neuromuscular paralysis and the human illness known as paralytic shellfish poisoning. Saxitoxin (STX) and tetrodotoxin (TTX) bind to a single site in the outer pore of the Na^+ channel, formed by the amino acid residues in the outer pore loops located between the S5 and S6 segments of each of the four homologous domains (I–IV) of the α-subunit (Fozzard and Hanck, 1996; Catterall, 2000; Zhang et al., 2013). The classic symptoms of PSP include vomiting, tingling of the lips, numbness of extremities, and breathing difficulties. Symptoms can occur within 30 min and death by respiratory failure can occur within 12 h (Halstead, 1978).

The level of PST bioaccumulation in shellfish varies widely among species (Bricelj and Shumway, 1998), which has been associated with differential sensitivity of nerves to STX (Twarog et al., 1972; Twarog, 1974). Twarog (1974) also theorized that recurrently exposed shellfish populations may undergo a genetic adaptation to PSTs with natural selection against individuals

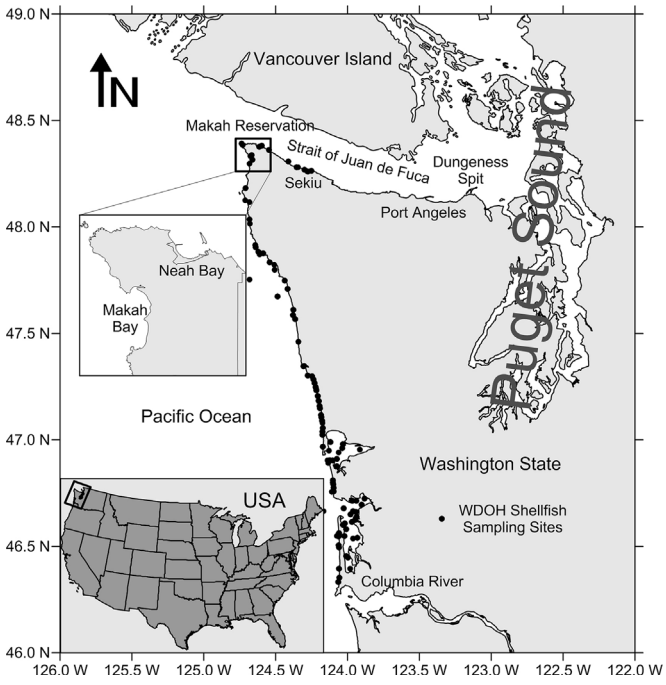

Fig. 1. Location of Makah Nation on the northwestern most portion of Washington State. The black dots indicate WDOH shellfish sampling sites from which PST data were obtained for use in this study (no data from locations east of Sekiu or Puget Sound are shown). The dots inside the box denoting the Makah Reservation refer to the "Makah Only" sites (data shown in Figs. 2, 3). PST test records spanned from October 1957 through October 2013. Species tested include: razor clam, California mussel, rock scallop, blue mussel, pacific oyster, butter clam, horse clam, Dungeness crab, littleneck clam, varnish clam, pink scallop, barnacle, cockle, manila clam, eastern softshell clam, hairy triton, weathervane scallop, and Olympia oyster.

sensitive to the toxins. Research on the soft-shelled clam (*Mya arenaria*) has shown an adaptation in clam populations from geographical regions with prior history of exposure to PSTs where the substitution of a single amino acid can confer resistance to STX (Bricelj et al., 2005, 2010; Connell et al., 2007; MacQuarrie and Bricelj, 2008). The specific substitution elucidated in *M. arenaria* was a simple change from adenine (A) to cytosine (C) in the domain II pore region of the alpha-subunit of the voltage-gated Na^+ channel (Bricelj et al., 2005). This modification caused a 1000 fold decrease in affinity of STX in Na^+ channels of resistant clams compared to sensitive clams with no prior history of PST exposure. This resistance mechanism is thought to allow clams to accumulate higher concentrations of PSTs (MacQuarrie and Bricelj, 2008).

Makah tribal folklore tells of potlatches (tribal parties) where visitors came over the mountains to eat seafood with locals. These stories suggested that the visitors became ill, while the Makah, who rely on shellfish in their diet, did not. One anthropological study reported that members of the Makah Tribe, located at the northwestern corner of Washington State (Fig. 1), believed that they were immune to shellfish poisoning due to their long history of exposure (Sepez, 2001). Shellfish are a major source of protein to the Makah Tribe and are the most widely consumed subsistence resource harvested in Neah Bay, where many Makah tribal members reside, and where almost 70% of households participate in harvest activities (Sepez, 2001). The Makah are aware of the risks of eating contaminated shellfish, but will often harvest from areas that they deem as safe, so incidents of PSP are rare.

The study investigated whether natural selection for resistant skeletal muscle Na^+ channels occurred in this community that has been chronically exposed to PSTs. It was hypothesized that natural selection for a Na^+ channel mutation could have occurred in ancestral Makah and passed down through generations to present day tribal members. Therefore, the expectations are to see prevalence of Na^+ channel mutations in the progeny of ancestral Makah. To this end, skeletal muscle Na^+ channel DNA was sequenced and questionnaires were distributed addressing ancestry and shellfish consumption in Makah tribal members.

2. Methods

2.1. PSTs in shellfish

Paralytic shellfish toxin test results were provided by the Washington State Department of Health (WDOH) to determine PSP risk in areas where members of the Makah tribal members typically harvest shellfish. Paralytic shellfish toxin data were acquired for the entire period of record (1957–2013) during which WDOH has been testing shellfish using the mouse bioassay. These data were divided into two groups to assess risk specific to the Makah Tribe. The first group included all of the WDOH sampling sites that were within the boundaries of the Makah reservation (Makah Only) and the second group included all of the sites on the WA State Pacific coast as well as sites in the Strait of Juan de Fuca (from Cape Flattery to Sekiu) where survey participants indicated they had harvested shellfish (WA coast excluding Makah, Fig. 1). The number of tests for PSTs, the number of positive tests (i.e. detectable levels of PSTs), percent positive tests, and the maximum PST concentration was determined for each group on a monthly, yearly, and species-specific basis to determine the PST exposure risk. To reflect a worst-case scenario when calculating average PST concentrations, a value of 30 μg STX eq./100 g shellfish meat was assigned to mouse bioassay tests (Association of Official Analytical Chemists, 1990) for which a "trace" concentration (i.e. toxic effects were observed but the concentration was not quantifiable) was

Table 1
Primers for PCR amplification and sequencing of fragments spanning the S5–S6 pore regions of the human skeletal muscle Na^+ channel. "F" denotes forward primer and "R" denotes reverse primer. (bp) = base pairs (n.b. Fragments 7 and 8 were amplified and sequenced as a single fragment with the appropriate sequence extracted after sequencing).

Fragment length (bp)	PCR primers	Sequencing primers	Fragment	Domain
1	F: CTCTGTTATCACCCTGCCCTGC R: TAAGAATGGGGAGGGTGAC	F: CTGCGGTCAGGGCTGAAGAC R: GCCTACATCAGTGATGAAGG	333	I
2	F: CAGGTGCTCACACTGTGT R: TTCCAGCTGGTAC	F: CATGTGGGTGACTTGCC R: GGACTATTGGGAGAACCTC	64	I
3	F: GGGGTCCCTATCTGCCCCCA R: TTGGCTCGGGGCAGGTGTCT	F: GGGGTCCCTATCTGCCCCCA R: TTGGCTCGGGGCAGGTGTCT	142	I
4	F: CTCTCTGATAACAGGATGCC R: GGAAAGGAGATAGAGGACC	F: CAGAACATGGCTCAGCATGCC R: GGAGAAGGTCTGGACTCAG	210	I
5	F: TACGCTTCCCGGGTGAGGGC R: GGCTGCCTTGGGTGGTGGTC	F: TACGCTTCCCGGGTGAGGGC R: GGCTGCCTTGGGTGGTGGTC	357	II
6	F: GAGGCACTGGCAATGGACC R: GCCGGGAACCTGGATGGAGG	F: GAGGCACTGGCAATGGACC R: GGTGGTGAGTGTGACCCACC	279	III
7	F: CTCTCAGGCCACCTTCAAGGGT R: GAAGAAGATGAGTATCAGCCC	F: CCTTCAAGGGTTGGATGGAC R: GAAGAAGATGAGTATCAGCCC	54	III
8	F: CTCTCAGGCCACCTTCAAGGGT R: GAAGAAGATGAGTATCAGCCC	F: CCTTCAAGGGTTGGATGGAC R: GAAGAAGATGAGTATCAGCCC	138	III
9	F: CCAGAAGTACTTCGTGTCACC R: GGAGAACTTCAATGTGGCC	F: CACGCTGTTCCGTGTGATCC R: GGTCAACATGTACATCGCC	454	IV

reported. A value of 30 μg STX eq./100 g reflects a concentration that is one unit lower than the lowest concentration reported in the data set. For reference, the regulatory action level for PSTs in Washington State is 80 μg STX eq./100 g shellfish meat.

2.2. Study participants

All activities complied with the requirements for human subjects research stated in 45 Code of Federal Regulations Part 46 (45 CFR 46). The scope of the research was explained to each participant who signed a Research Consent Form, which acknowledged their understanding of the research and provided their written, informed consent to participate. The identity of each participant was protected by assigning a random four-digit number to code all survey responses and DNA samples. Survey questions included age, gender, family history in the Makah Tribe, residence on the Makah reservation, and shellfish consumption habits in the Fall/Winter (September through February) and Spring/Summer (March through August). This study was approved by the Institutional Review Board of the University of Maryland, Baltimore. All investigators completed the training required for studies involving human subjects.

2.3. Sample collection and extraction

Study participants were instructed to abstain from fluids other than water for 2 h prior to DNA sample collection. Immediately prior to sample collection, the study participants were asked to rinse their mouths twice with water. After rinsing, cheek cells were collected using sterile Catch-All™ Sample Collection Swabs (Buccal Amp DNA Extraction Kit, Epicentre Biotechnologies). The swabs were gently rolled on the inside of the cheeks of each participant approximately 5 times then air dried for 10 min. The tip of the swab used to collect the cheek cells was cut off and placed into a tube of QuickExtract™ DNA Extraction Solution 1.0 (Buccal Amp DNA Extraction Kit, Epicentre Biotechnologies). The tube was stored at 4 °C at the sampling location and subsequently transported on ice to the laboratory for DNA extraction following the manufacturer's protocol. Briefly, the tube was vortexed for 10 s followed by incubation at 65 °C for 1 min. The tubes were then vortexed for an additional 15 s and incubated at 98 °C for 2 min. Finally, the tubes were vortexed for 15 s and stored at 4 °C prior to polymerase chain reaction (PCR) and sequence analysis.

2.4. PCR and sequencing

Eight PCR primer sets (Table 1) were designed *in silico* to amplify exons that code the membrane spanning S5–S6 regions of domains I–IV (Fig. 4) in the human skeletal muscle voltage-gated Na^+ channel gene (SCN4A, NCBI accession number NM000334). Amplifications were conducted in 20 μL volumes containing 2 μL template DNA, 312.5 nM of each primer, 12.5 mM Tris–HCl (pH 8.5), 62.5 mM KCl, 0.125% Triton X-100, 2.5 mM of each dNTP, 1 unit *Taq* polymerase (Apex Bioresearch Products), 1.875 mM $MgCl_2$, and sterile ddH_20. Each fragment was amplified using a Bio-Rad iCycler thermocycler (Bio-Rad Laboratories, Hercules, CA) with an initial denaturation step of 94 °C for 2 min followed by 35 cycles of 95 °C for 30 s, 60 °C (56 °C for fragments 3 and 6, see Table 1) for 30 s, and 72 °C for 90 s, and a final extension of 72 °C for 5 min followed by a 4 °C hold.

PCR products were purified prior to sequencing using Millipore Multiscreen PCR plates (EMD Millipore Corporation, Billerica, MA). Sterile ddH_2O (100 μL) and corresponding PCR products (20 μL) were added to each well. The filtration plate was placed on a Millipore vacuum manifold (Millipore Corporation, Hayward, CA) and a pressure of 22 in Hg was applied until all of the liquid had been drawn through the membranes (~5–10 min). After all of the wells were empty, the pressure was released and any liquid on the bottom of the plate was blotted with a paper towel. Forty microliters of sterile ddH_2O was added to each well, the wells were sealed, and the plate was placed on a shaker platform at the lowest setting for approximately 15 min to resuspend the PCR product. The purified PCR products were removed from the Millipore plate and transferred to a 96 well storage plate and kept at 4 °C.

Forward and reverse sequencing reactions (1/8 reactions) were performed on the purified PCR products using the Applied Biosystems (Foster City, CA) BigDye® Terminator v3.1 Cycle Sequencing kit. Each 10 μL reaction consisted of 1 μL Terminator Ready Reaction Mix, 3.25 pmol of primer, 2.0 μL PCR product

Table 2
Washington State Department of Health PST test results from 1957 to 2013. Data were separated into 2 groups: "Coast excluding Makah" which included sites outside of the Makah reservation on the outer coast and along the Strait of Juan de Fuca and "Makah Only" which included sites only within the Makah reservation boundary (see Fig. 1). The regulatory action level for PSTs is 80 μg STX eq./100 g shellfish.

Species	Coast excluding Makah			Makah Only		
	Max. PST	Positive tests	# Tests	Max. PST	Positive tests	# Tests
Barnacle	42	1	2	na	na	na
Blue mussel	720	139	651	1096	57	77
Butter clam	132	7	12	116	14	36
California mussel	1603	538	1854	1169	150	334
Cockle	0	0	3	40	2	2
Dungeness crab	72	1	10	na	na	na
Eastern softshell clam	0	0	1	na	na	na
Hairy triton	0	0	1	na	na	na
Horse clam	122	1	7	56	4	21
Littleneck clam	71	2	64	56	1	17
Manila clam	39	1	150	na	na	na
Olympia oyster	0	0	1	na	na	na
Pacific oyster	356	497	4490	na	na	na
Pink scallop	48	2	2	na	na	na
Razor clam	3480	1410	4218	59	1	3
Rock scallop	562	17	19	1505	14	15
Varnish clam	na	na	na	50	3	7
Weathervane scallop	0	0	3	na	na	na

na = species not tested.

template, 2.0 μL 5× Big Dye Sequencing buffer, and 4.87 μL sterile ddH_2O. Sequencing reactions were carried out on a Bio-Rad iCycler thermocycler using a ramp rate of 1 °C per second and a thermal profile of 25 cycles that consisted of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min, followed by a 4 °C hold step. Unincorporated dye was removed from the reactions using Agencourt CleanSEQ (Agencourt Bioscience Corporation, Beverly, MA) following the manufacturers protocol and sequencing was carried out on an ABI 3100 (Applied Biosystems, Foster City, CA, USA).

2.5. Sequence editing and alignment

Forward and reverse nucleotide sequences were aligned using Geneious Pro Version 5.0.4 (Biomatters Limited, Auckland, New Zealand). Each alignment was manually edited to resolve ambiguities. The consensus nucleotide sequences for each exon were aligned and compared to the reference sequence from NCBI (NM000334) to assess differences. The nucleotide sequences were translated into their corresponding amino acid sequences and aligned with the translated reference sequence to evaluate substitutions.

2.6. Family history categories

The data from survey questions pertaining to Makah tribal membership of the parents, grandparents (maternal and paternal), and great-grandparents (maternal and paternal) of participants were sorted in the order in which they appeared on the survey and grouped to construct a family history composite string variable for each study participant. All survey questions were answered by the participants and there were no missing data. Unique composite family histories were assigned nominal categorical values from 1 to *k*, where category number 1 is made up of individuals for whom all combinations of parents, grandparents, and great-grandparents were Makah tribal members and category *k* is made up of individuals with no family history of Makah tribal membership. The categories between 1 and *k* are comprised of various unique patterns describing the Makah tribal membership of the parents, grandparents, and great-grandparents of the study participants. Similarly, unique Fragment 1 amino acid sequences were assigned nominal values depending on the observed amino acid substitutions after translation.

3. Results

3.1. PSTs in Makah harvest region

Paralytic shellfish toxins have been detected even when the blanket harvest closure each year from 1 April to 31 October from the Columbia River to Dungeness spit (Fig. 1) is not in effect. Washington State Department of Health data for the outer coast of Washington State and the western portion of the Strait of Juan de Fuca show a wide range of detectable PSTs in a variety of shellfish (Table 2) harvested from the Makah reservation and other coastal sites at all times of year (Fig. 2A and B). A greater number of tests were performed on certain species of shellfish, such as razor clams (*Siliqua patula*), Pacific oysters (*Crassostrea gigas*), California mussels (*Mytilus californianus*), and blue mussels (*Mytilus trossulus*) than others, but even those species with relative few tests such as butter clams (*Saxidomus giganteus*) and rock scallops (*Crassadoma gigantea*) had maximum PST concentrations well above the regulatory action level of 80 μg STX eq./100 g shellfish meat (Table 2). These data also show that PST concentrations are present in WA coastal waters during all months of the year (Fig. 2A and B). Moreover, the maximum PST concentration in half of the shellfish species with detectable PSTs exceeded the regulatory action level (Table 2). Mussels and razor clams, two types of shellfish consumed in the greatest numbers by study participants, had the highest maximum concentrations of PSTs (Tables 2 and 3).

Tests for PSTs in shellfish have been conducted in most years for the period of record and testing has become more frequent since the 1980s (Fig. 3A and B). There were no PST tests performed by WDOH within the Makah reservation for some years during the period of record (Fig. 3A), including the time period (2007–2008) for which study participants were asked to provide information on their shellfish consumption. The yearly maximum PST concentrations observed for both the Makah reservation (Fig. 3A) and the other coastal sites (Fig. 3B) ranged from no toxin detected, mostly for years where few samples were analyzed, to well over the regulatory action limit (e.g. 3480 μg STX eq./100 g in razor clams).

3.2. Study participant shellfish consumption

The study participants were asked about their general shellfish consumption and consumption of specific shellfish types during

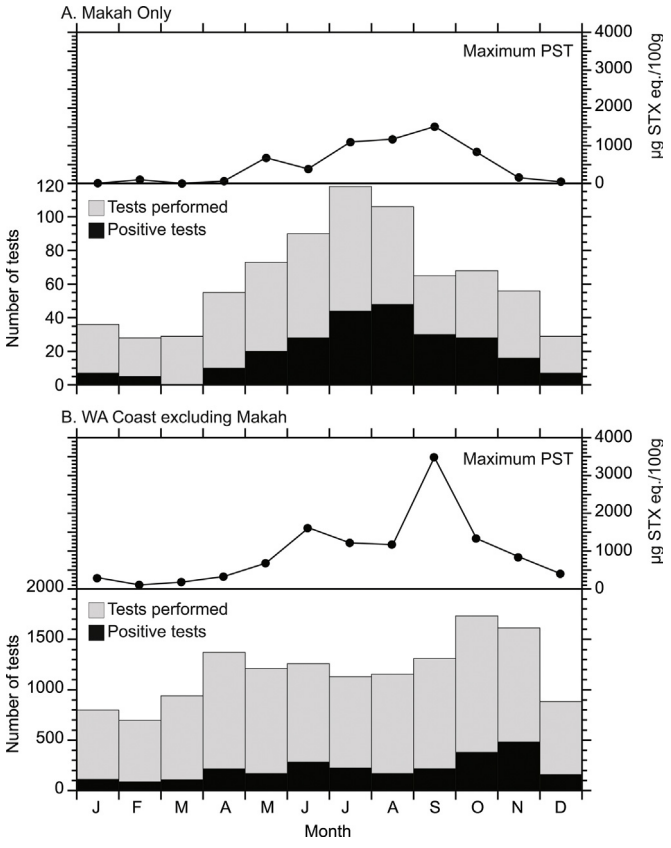

Fig. 2. Monthly record of tests with detectable PST concentrations (“positive tests”), the number of tests that were performed, and the maximum PST concentration measured for the period of record (October 1957–October 2013). “Makah Only” (A) refers to sites within the Makah reservation boundary (see Fig. 1) and “WA Coast excluding Makah” (B) sites noted with solid dots (Fig. 1) outside of the Makah reservation boundary. Note the differences in scale for number of tests for each panel. The regulatory action level for PSTs is 80 μg STX eq./100 g shellfish meat. All monthly maximum PST values were above the regulatory action level except for January, March, and April at Makah Only sites, and all months had positive PST tests except for March at Makah Only sites.

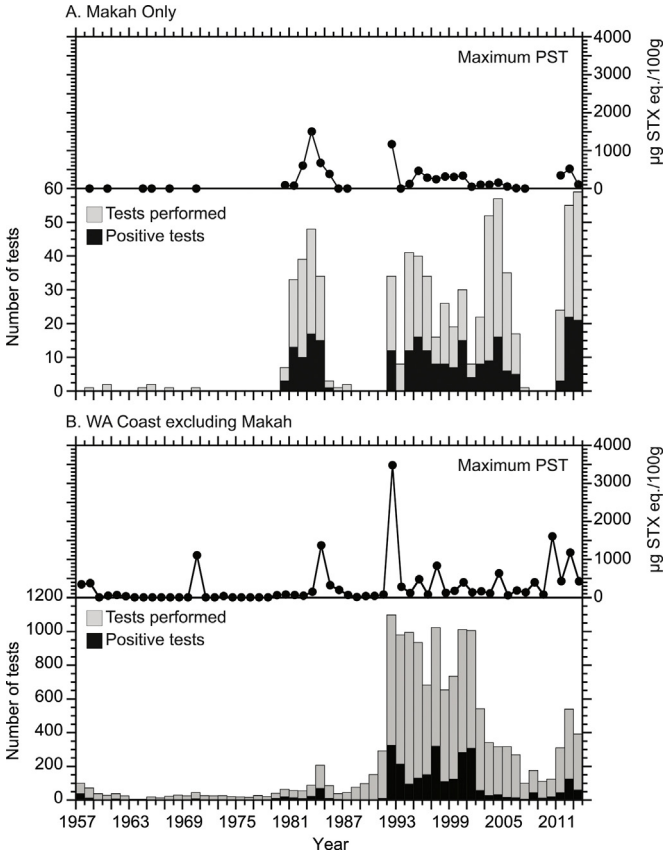

Fig. 3. Annual record of tests with detectable PST concentrations (“positive tests”), the number of tests that were performed, and the maximum PST concentration measured for the period of record (October 1957–October 2013). “Makah Only” (A) are sites within the Makah reservation boundary and “WA Coast excluding Makah” (B) are sites noted with solid dots (Fig. 1) that are outside of the Makah reservation boundary. Note the differences in scale for the y-axis in A and B. In (A) the line in the maximum PST panel is not continuous due to missing data. The regulatory action level for PSTs is 80 μg STX eq./100 g shellfish meat.

Fall/Winter 2007 and Spring/Summer 2008 on separate surveys (Table 3). Seventy six of the 83 participants provided information about their consumption of cockle clams (*Clinocardium nuttallii*), geoduck clams (*Panopea generosa*), Manila clams (*Venerupis philippinarum*), crabs (*Cancer magister*, *Cancer productus*), and scallops (*Crassodoma gigantea, Patinopecten caurinus*). Seventy five of the 83 participants provided information regarding consumption of razor clams (*Siliqua patula*), mussels, littleneck clams (*Protothaca staminea*), butter clams, horse clams (*Tresus nuttallii*), chitons (*Katharina tunicata*, *Mopalia mucosa*), gooseneck barnacles (*Pollicipes polymerus*), and moonsnails (*Neverita lewisii*). Between one and 53 participants indicated they consumed some type of shellfish in the Fall/Winter and between one and 37 participants consumed some type of shellfish in the Spring/Summer. All shellfish types were consumed to varying degrees in the Fall/Winter and Spring/Summer. None of the participants indicated that they had become ill after eating razor clams, mussels, or crabs, however, participants were not asked about becoming ill after eating other types of shellfish.

3.3. Samples and sequencing

Cheek cells were collected from a total of 83 individuals for this study. For each fragment, the number of successful alignments and the number of sequences with single nucleotide polymorphisms (SNPs) ranged from 76 to 83 and 0 to 23, respectively, with multiple SNP forms for some of the fragments (Supplemental Table S1). Two of the 23 individuals with SNPs in Fragment 9 were homozygous, whereas individuals with SNPs at all other loci were heterozygous. Fifty of the composite nucleotide genotypes had no mutations, 22 had one mutation, nine had two mutations, and two had three mutations. Thirty-nine individuals shared a single genotype with between two and eight individuals sharing six other genotypes. Thirteen individuals had genotypes that were not shared with others. Overall, there were 20 unique nucleotide genotypes.

After translation, five Fragment 1 sequences contained single amino acid substitutions. Three of the five Fragment 1 sequences with differences had substitutions at position 73 [glycine (G) → aspartic acid (D)] and two sequences had substitutions at position 88 [threonine (T) → methionine (M)] (Fig. 5). The substitutions in the Fragment 1 sequences occurred in the Na^+ channel pore loop region in the extracellular portion of the channel (Fig. 4). In total, there were three forms of the Fragment 1 amino acid sequence (identical to translated reference sequence, G → D substitution, and T → M substitution). All of the translated sequences for Fragments 2–9 were identical to the translated reference sequence (Supplemental Table S1).

3.4. Demographics

Of the 83 individuals from whom cheek cells were collected, 73 provided family history information detailing whether or not

Table 3
Fall/Winter (2007-2008) and Spring/Summer (2008) shellfish consumption data for study participants. The mean number of shellfish consumed is the average number eaten by participants who consumed this particular shellfish species during each time period.

Shellfish	Fall/Winter		Spring/Summer	
	Participants who consumed shellfish	Mean number of shellfish consumed	Participants who consumed shellfish	Mean number of shellfish consumed
Cockles	31	5.2 (3.7)	27	6.6 (4.5)
Geoduck	13	3.2 (2.9)	8	4.0 (3.5)
Manila clam	24	6.9 (5.5)	22	7.3 (8.4)
Crabs	53	2.0 (1.8)	37	1.5 (0.8)
Scallops	15	5.4 (4.7)	12	6.3 (5.3)
Razor clams	30	5.3 (4.4)	25	4.4 (4.0)
Mussels	26	4.8 (3.3)	21	5.0 (3.7)
Littlenecks	21	9.6 (7.3)	13	10.0 (6.7)
Butter clams	29	8.3 (8.4)	12	7.7 (4.8)
Horse clams	11	4.7 (2.5)	8	4.1 (2.0)
Chiton	8	5.8 (4.4)	4	6.8 (3.4)
Gooseneck barnacles	9	6.9 (6.3)	4	5.3 (2.9)
Moonsnails	1	2.0 (0.0)	1	2.0 (0.0)

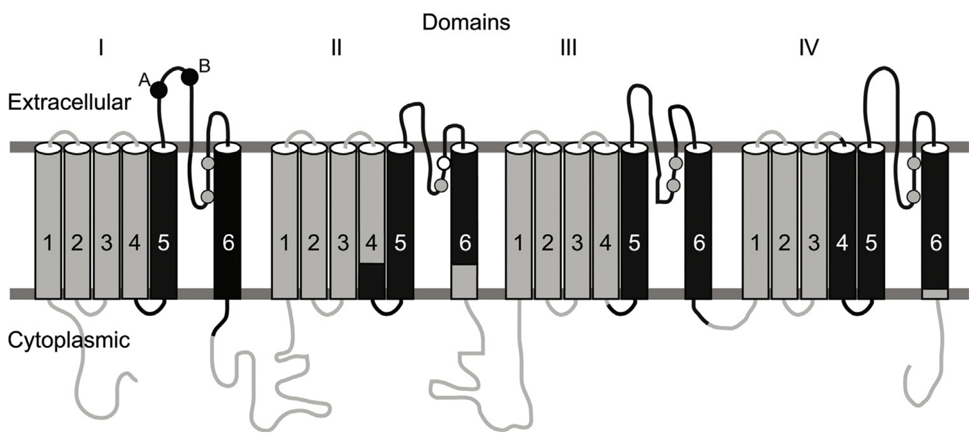

Fig. 4. Domains I–IV of the human skeletal muscle Na^+ channel, with regions sequenced for this study shown in black. Cylinders represent transmembrane regions and solid lines represent extracellular regions. The circles on the pore loops between transmembrane regions 5–6 of each domain represent the approximate locations of residues critical for STX binding. The location of an amino acid mutation in *M. arenaria* responsible for conferring STX resistance is shown as an open circle in domain II. The approximate locations of the amino acid substitutions found in five Makah tribal members are in domain I (solid circles A, B). Three individuals had substitution A (G to D) and two individuals had substitution B (T to M). A portion of this region is shown in Fig. 5.

their parents/grandparents/great-grandparents were Makah tribal members and 24 unique composite family histories were generated. Additionally, a majority of the participants (61) indicated that their predecessors lived on the reservation for 80 or more years and the overall range was 0–102 years. Participants ranged from 21 to 83 years of age at the time of the study and indicated that they had lived on the reservation from 3 to 83 years with a majority (63) having spent more than 50% of their lives on the Makah reservation.

The study participants for whom Fragment 1 amino acid substitutions were observed ranged in age from 26 to 64 years old. Four of these participants were male, one was female, and all were

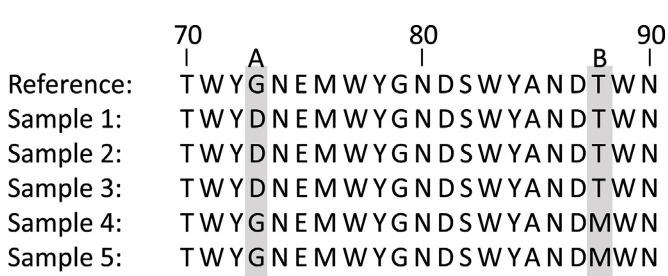

Fig. 5. A portion of the Fragment 1 amino acid sequence showing the observed substitutions (A and B, Fig. 4) highlighted in gray.

registered members of the Makah Tribe. These study participants had lived on the Makah reservation from 3 to 64 years and indicated that their predecessors had lived on the reservation from 33 to 100 years. There were four nucleotide genotypes and four aggregate family histories represented in this group of five individuals. The fathers of the three individuals with the G to D substitution in Fragment 1 were not Makah tribal members nor were their paternal grandparents or great-grandparents. The mothers, maternal grandmothers and maternal great-grandmothers of these three individuals were all Makah tribal members. In two of the three individuals with the G to D substitution, the maternal grandfathers and maternal grandfathers were also Makah tribal members. The parents, grandparents (both maternal and paternal), and great-grandparents (both maternal and paternal) of one individual with the T to M substitution in Fragment 1 were Makah members whereas only the father, paternal grandfather, and paternal great-grandfather of the other individual were Makah members. The family histories of this group were not unique in that the same histories were also observed in individuals that had no amino acid substitutions in Fragment 1. Further, a plot of Fragment 1 amino acid categories vs. family history categories showed that family histories were shared among amino acid sequence categories (Fig. 6) suggesting that family history had no bearing on the incidence of the observed amino acid mutations.

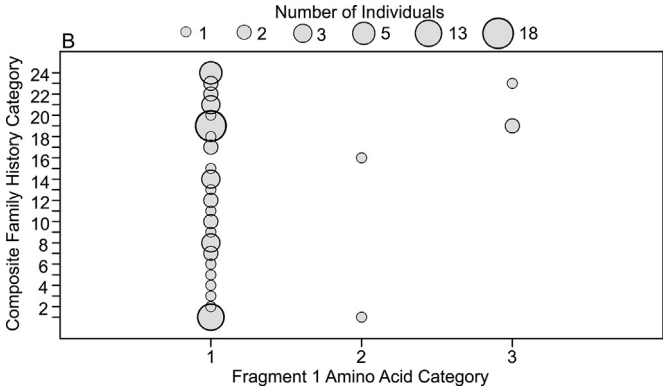

Fig. 6. Relationship of Fragment 1 amino acid categories composite family history categories. The size of the bubble denotes the number of individuals with particular family history that also exhibited specific Fragment 1 amino acid substitutions. Amino acid category 1 denotes individuals with sequences that are identical to the reference sequence from NCBI (NM000334), category 2 are individuals with the T → M substitution, and category 2 are individuals with the G → D substitution. Family history category number 1 is made up of individuals where all combinations of parents, grandparents, and great-grandparents were Makah tribal members and category 24 is made up of individuals with no family history of Makah tribal membership. Family history categories 2–23 are comprised of various unique combinations of Makah membership for parents, grandparents, and great-grandparents of study participants.

4. Discussion

Makah tribal members have stated that they repeatedly eat shellfish during closure periods and believe that they are resistant to PSTs. This belief has some credence, due to the fact that a PST resistance mutation has been mapped in *M. arenaria* from areas that experience repeated problems with PSTs (Bricelj et al., 2005). The goal of this study was to determine whether or not a mutation in the human skeletal muscle Na^+ channel was present in the relatively geographically isolated Makah tribal population with recurrent historical exposure to PSTs. We theorized that the longer a family lived on the reservation, including their more distant ancestors, exposure to PSTs would have been heightened and the chances that selection for a Na^+ channel mutation that could confer resistance to PSTs would have increased. The skeletal muscle Na^+ channel was chosen for sequencing because respiratory muscle paralysis is a classic sign of PSP and one of the most significant causes of PSP-related death in humans.

4.1. Paralytic shellfish poisoning risk assessment

The Washington State coast from the Columbia River to Dungeness Spit (Fig. 1) is closed to molluscan shellfish harvesting yearly from 1 April to 31 October as a mitigation measure for PSP with an exemption for razor clam harvesting only on designated open beaches. However, detectable PST concentrations as well as PST concentrations above the regulatory limit have been observed between 1 November and 31 March at locations within the Makah reservation, as well as other locations on the WA outer coast (Fig. 2A and B). Therefore, those shellfish harvesters who collect shellfish during the seasonal closure period on the Washington State coast are at risk for PSP.

Depending on when and where these shellfish are harvested, there is a substantial PSP risk to Makah tribal members. Shellfish collected for PST testing by WDOH at locations within the Makah reservation show that PST levels can exceed the regulatory action level throughout most of the year. Similarly, the survey results indicated that Makah participants did not alter consumption of shellfish during the year (Table 3), putting them at risk for PSP. The Washington State shellfish toxin data clearly indicate a historical PSP exposure risk. Although fewer positive tests were reported from 1957 to the beginning of the 1990s, this reflects the 1 April–31 October coastwide closure, when testing was not routinely conducted, rather than lack of PSTs in shellfish during this time. Likewise, in years where shellfish toxin testing was not performed from the Makah reservation (Fig. 3A), levels at adjacent sites confirm the presence of PSTs in shellfish harvested from the region.

4.2. Na^+ channels and toxins

Some human muscle diseases, such as hyperkalemic periodic paralysis and myotonia, have been attributed to mutations in the skeletal muscle Na^+ channel (Catterall, 1992; Petitprez et al., 2008). Additionally, point mutations in Na^+ channels have been shown to confer resistance towards toxins (e.g. STX, TTX) that alter their normal function (Noda et al., 1989; Satin et al., 1992; He et al., 1999; Geffeney et al., 2002; Bricelj et al., 2005). Portions of the human skeletal muscle Na^+ channel (Fig. 4) were sequenced from Makah participants to look for point mutations that could confer resistance to STX in this population. Single amino acid mutations were observed in the extracellular regions between S5 and S6 of domain I in the skeletal muscle Na^+ channel in five of the 83 Makah tribal members (Fig. 4). However, this region has not been associated with STX binding (Yu and Catterall, 2003; Zhang et al., 2013), so it would be unlikely to confer STX resistance. If a Nav1.4 mutation cannot explain perceived resistance to STX by Makah tribal members, it may be that a mutation is present only in the neuronal Na^+ channel and not skeletal muscle channels. However, because the primary symptoms of PSP include muscle and nerve paralysis, it is hypothesized that if a resistance mechanism exists in humans, relevant mutations should be observed in both types of Na^+ channels.

In softshell clam populations that contain nerves resistant to PSTs, recurrent annual toxic outbreaks of the dinoflagellate genus *Alexandrium* cause natural selection for the single point mutation in domain II of the Na^+ channel gene (Bricelj et al., 2005). This resistant genotype can be homozygous or heterozygous at the identified locus, and more than one nucleotide substitution can result in resistance (Connell et al., 2007). This natural selection for resistance varies by latitude with the intensity, timing and duration of toxic algal blooms along the Atlantic coastal area of softshell clam distribution (from the northern U.S. to Canada). Such a difference in the resistance to PSTs relative to the prior history of algal blooms has been observed in the copepod, *Acartia hudsonica*, along the U.S. east coast (Colin and Dam, 2003, 2004). However, a Na^+ channel mutation at the STX binding site has not been observed in these copepods (Chen, 2010) and the mechanism of resistance is not yet understood.

Natural selection for resistance likely occurs at times and in regions when juvenile clams are exposed to highly toxic blooms for a substantial period of time. Exposure of early life-stage softshell clams to highly toxic *Alexandrium tamarense* cells has a selective effect for clams with the STX resistant Na^+ channels, whereas shorter exposure to more modest concentrations of toxins likely leads to paralysis and inhibition of growth rather than death and concurrent selection of resistant populations (Bricelj et al., 2010). However, the generation time for humans is much longer than for clams, therefore a human population would likely need to be exposed to high PST concentrations for a much longer time period to allow selection for a resistant sodium channel. While PSTs have only been documented in the Pacific Northwest for the last 200+ years, they likely have been present in this region for much longer. Moreover, death due to PSP in humans is a relatively rare occurrence, at least in modern times. Except for the PSP deaths in 1942 in Sekiu, WA, 30 km east of the Makah reservation, no other deaths have been recorded in Washington State. A resistance genotype likely is not conferred to humans exposed to shellfish with moderate toxin concentrations for the same reason that clams

from regions with modest concentrations of PSTs do not show the resistance genotype (Bricelj et al., 2010).

Greater than 250 cases of PSP were recorded in the Bay of Fundy, eastern Canada from 1689–1970 (Prakash et al., 1971), pointing to the fact that this site, where resistant clams have been found, is a toxic "hot spot". The most commonly eaten clams in eastern Canada during that same period were *M. arenaria* with the highest recorded PST concentrations of 6600–7700 μg/100 g. The highest recorded concentration of PSTs in the blue mussel (*Mytilus edulis*) was 28,000 μg/100 g in 1944. These toxin concentrations more than 18 times higher than the maximum concentration of 1505 μg/100 g observed in shellfish on the Makah reservation. The average concentration of recorded PSTs at the Makah reservation were estimated at 53 μg/100 g per day (only those days when toxin measurements in shellfish were made), well below the estimated concentration of 200–1000 μg known to cause death in humans (Tennant et al., 1955; Schantz, 1970). We theorize that the historical concentrations of PSTs in shellfish were not consistently high enough, or years of exposure not long enough, to confer Na^+ channel gene resistance to the Makah.

4.3. Avoidance harvesting and PSP

The Makah may use shellfish harvesting behaviors that help them avoid exposure to PSTs (Sepez, 2001). They may harvest at times of year when shellfish are the least likely to contain levels of PSTs above the regulatory limit. However, WDOH data show that PSTs can be present year round, at least since 1992 (Fig. 2). Additionally, environmental observations and local traditional knowledge may provide clues that shellfish are "no good" (Sepez, 2001). This may include an understanding that certain species of shellfish can retain toxins for longer periods of time (Bricelj and Shumway, 1998), but this behavior is not specifically identified by Sepez (2001) as a mechanism of harvest avoidance.

While local knowledge and avoidance harvesting may mitigate acute cases of PSP, chronic low-level exposure to PSTs may be a risk to tribal members, potentially triggering an immune response. In laboratory studies, an antibody response has been reported in zebrafish exposed to sub-lethal concentrations of another common seafood toxin, domoic acid (Lefebvre et al., 2012). The antibody response was indicative of chronic low-level exposure and increased neurologic sensitivity to domoic acid. To date, such an antibody response has not yet been shown for saxitoxin, but, if present, would be a valuable risk assessment tool for chronic exposure to PSTs in humans and other organisms.

5. Conclusions

The members of the Makah Tribe that harvest shellfish are an at-risk population for PSP. Amino acid mutations were observed in the skeletal muscle extracellular region between S5 and S6 of domain I in some individuals, but not in the STX binding region. The location of this mutation is not at the known site of STX binding, therefore the Na^+ channel mutation in these individuals does not confer resistance to PSTs. The observed amino acid mutations were not unique to any particular pattern of Makah family history. Although no significant mutations were seen in the skeletal muscle Na^+ channel, it is possible, but not likely, that other human Na^+ channels, such as neuronal (Nav1.1) and cardiac (Nav1.5), contain a resistance mutation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.hal.2016.03.008.

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