Identifying Genetic And Environmental Risk Factors For Chronic Orofacial Pain Syndromes: Human Models

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Chronic orofacial pain syndromes are produced by nerve injury, diseases, and toxins. They constitute an unsolved medical problem because they affect a considerable number of adults and are difficult to treat. There is a remarkable variability among adults in terms of susceptibility to chronic orofacial pain and its characteristics, which suggests that these syndromes are complex heritable traits controlled by alleles of certain polymorphic genes that interact with the environment. Each syndrome is assumed to be determined by a unique set of genes. In the present report, a practical study design is proposed to identify the genes responsible for interindividual variability in orofacial pain levels. This design is based on research strategies that have been used for studying other human diseases as well as pain syndromes outside the orofacial region. Specifically, this design has been used successfully by the authors and others over the past 8 years to study chronic pain syndromes such as migraines, radiculopathy, amputation pain, and postmastectomy pain. The strategies used to study these topics have been adapted to address the unique problems of orofacial pain. The authors believe that the study of genetics provides a novel research approach from which to identify targets for the development of individually tailored approaches in orofacial pain medicine, such as diagnostic and prognostic kits and novel drugs that would prevent pain chronicity in susceptible individuals or alleviate it once it had developed. This report focuses on human models. A follow-up report is intended to extend this design into animal models of orofacial pain syndromes. J OROFAC PAIN 2004;18:311–317

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Chronic orofacial pain syndromes include many conditions where pain is the main symptom; in some conditions, it is the only symptom. These syndromes are produced by nerve injury, diseases, and toxins.[1,2] They usually involve much suffering, physical incapacitation, loss of work, depression, and negative effects on family and social life. Chronic pain is a crucial societal issue with a huge economic burden. Not only is chronic pain very common, but it is also generally difficult to treat.[3,4] Some orofacial syndromes have a higher prevalence in women compared to men, yet current research still fails to address this peculiarity. For greater detail on the clinical aspects, classification issues, and treatment of orofacial pain syndromes, the reader is referred to the reports of Bennett,[1] Zakrzewska,[2] Truelove,[3] and Watson[4] in this issue of the *Journal of Orofacial Pain*.

The failure of our current clinical approaches to treat these pain syndromes[3,4] perhaps calls for no less than a paradigm shift in our approach to the understanding of chronic pain and its therapy. It is believed that pain genetics may provide such a shift, since

genetic as well as environmental factors may be involved in the development of several pain conditions, including those in the orofacial region. This report provides a practical guide on how to engage in a project on genetic and environmental aspects of orofacial chronic pain.

Why Engage in Genetics of Orofacial Pain Now?

Not every individual exposed to the same chronic pain–producing etiologic factor eventually develops chronic pain.[1,2] Moreover, there is a remarkable variability in pain levels among those individuals who develop orofacial pain syndromes. Each patient presents a unique combination of pain descriptors such as "burning," "electric shock–like," etc. The intensity, location, duration, and frequency of a typical pain episode and the impact of pain on quality of life and daily activities are also highly variable from individual to individual, and so are responses to analgesic drugs.[1–4] This variability and the predominance of females presenting with most chronic orofacial pain syndromes are compatible with the hypothesis that the susceptibility to pain syndromes is a heritable complex trait determined by a combination of alleles of polymorphic genes and environmental variables. Studies on the incidence of trigeminal neuralgia[5] and familial migraine[6] in twins and pedigrees corroborate this hypothesis. A few reports have already identified genetic determinants affecting chronic pain in these syndromes.[6–8] More support for this hypothesis can be found in reports on heritability of chronic pain syndromes in body parts other than the trigeminal system and the head.[9–16] For several syndromes there are reports on the chromosomal region where pain genes are located[17–20] and even identified genetic polymorphisms associated with pain levels.[21,22] Many studies have already established that levels of chronic pain in animal models of neuropathies are heritable.[23–26] On the basis of these reports and the recent progress made in the Human Genome Project, it is now time to extend these observations to orofacial pain syndromes in humans. The following provides an outline of how this might be accomplished.

Study Design for Genetics/Phenomics of Chronic Orofacial Pain

The following 8 steps outline research approaches and methodologies to be considered when undertaking such a project.

Step 1: Select a Suitable Pain Syndrome for the Project

For statistical power, one needs to recruit many hundreds of genetically unrelated pain patients with the same syndrome as well as matching controls.[27–29] The latter subjects should be patients who have undergone the same surgery, disease, or trauma, but have not developed chronic pain, and who are matched for age, etiology, ethnicity, and sex. While collecting these subjects in an "association study design," which contrasts genetically unrelated subjects,[28] it is advisable to inquire whether other individuals in the family suffered from any type of chronic pain, especially orofacial, and whether they are consanguineous (ie, have shared genes) or related by marriage (ie, have a shared environment). Coming across nuclear (consanguineous) families with several pain patients may enable a genetic "linkage study design"[28] to be used. In certain favorable cases, several such families may be enough to identify pain genes.

A cohort size of many hundreds of cases is only sufficient to detect genes having a major effect on pain levels and alleles that are frequent in the population. Identifying rare alleles or genes having a smaller effect on trait variance ("modifier genes") necessitates a considerably larger study group.[28] Genotyping results of an association study are additive to the results others obtain for the same genotypes and phenotypes, thus increasing the statistical power for a meta-analysis.[28]

Not every patient invited to participate in a study on pain also consents to participate in a genetic study, which requires the patient to spend hours on detailed phenotyping questionnaires and to return for additional sessions if a longitudinal study calls for it. Typically, our studies are based on about 60% of the pain patients and 40% of the control subjects who were originally invited to enroll to the study. Therefore, a study cohort that reports on 300 pain patients and 300 matched controls should begin with the availability of about 500 pain patients with the chosen syndrome and about 800 controls. This cohort size calls for a multicenter study design and 2 to 3 years to collect the desired cohort size. Thus, the sooner patient recruitment is begun, the better.

Step 2: Collect Evidence that the Chosen Orofacial Pain Syndrome is Heritable

It is advisable to carry out a twin study and/or pedigree analyses to establish the level of heritability of the pain trait under investigation. This value

estimates the part of the trait variance that heredity may account for. Similar studies estimated heritability in a number of chronic pain syndromes ranging from 40% to 60% of the trait variance. This range suggests that drugs targeting the product of pain genes could have a significant effect on pain levels.[30]

Step 3: Define the Pain Phenomes Characterizing the Chosen Orofacial Pain Syndrome

Chronic pain in humans is a multidimensional experience, comprising sensory-discriminative, affective, and cognitive facets and affected by situational and hormonal variations, mood, and the effects of previous encounters with chronic pain. Therefore, to faithfully represent the experience of chronic pain, one would optimally need to collect as many phenotypes characterizing the chosen syndrome as possible.[27] Ascertaining the pain phenotype is important. Therefore, if technically possible, assessing the pain traits in the same subjects twice over a period of several weeks provides an opportunity for "test-retest" validation of the levels of pain reported by the participants.

A genotype under study is then sequentially tested for segregation against each collected phenotype in a search for statistically significant associations. Thus, genes for a certain "pain syndrome" are nothing more than genes for the phenotypes tested. However, collecting many phenotypes has several disadvantages. First of all, loading participants with several questionnaires, each comprising many questions, puts their cooperation at risk. Secondly, if multiple statistical segregation tests of the same genotype against many phenotypes are run, the alpha level must be adjusted to minimize the occurrence of type 1 statistical errors that may falsely identify genes as relevant to orofacial pain. This adjustment is done by the use of the Bonferroni correction factor, by dividing the level of significance (usually set at $P \leq .05$) by the number of tests made. Thus, it does not take many comparisons to make the adjusted significance threshold so low that it becomes nearly impossible to cross it and find a significant segregation between any of the phenotypes and a genotype under investigation. This result, however, carries the risk of a type 2 statistical error, where genotypes that have a real effect on a pain phenotype will not be detected. If some of the tested phenotypes are partially correlated, a more lenient correction factor for multiple comparisons than the Bonferroni correction factor is justified.

One way of minimizing the number of statistical comparisons, and hence the need to adjust the alpha level, is to identify a subset of phenotypes that can be compressed into a single index. However, indexing carries the disadvantage of losing phenotypic robustness and resolution and thus reduces the chance of finding significant segregations. Alternatively, one may decide that this part of the study is an explorative, preliminary stage, avoid adjusting the alpha level, and declare that the results will be used as a basis for hypotheses to be tested in a follow-up study. In the next run, which would be carried out in a new cohort of subjects, only segregations found in the previous run to be statistically significant should be examined, and the alpha level for multiple comparisons should be adjusted promptly. Because only a few segregations will be tested in the replication run, the chance of finding significant correlations is high.

Step 4: Collect Evidence that Orofacial Pain Phenotypes are Modulated by Environmental Factors

Understanding the mechanisms of gene-by-environment interaction is important if eventually we are to provide our patients with a comprehensive solution that addresses both aspects of the trait variance—genetic and nongenetic. Any variable that affects chronic pain and is not caused by interindividual DNA sequence variations or gene-gene interactions is regarded as "environmental." These include internal and external environmental pain modifiers. Several clinical studies have already characterized a number of such variables.[31,32] However, a series of studies is needed that systematically detail the effects of diet,[33] social interactions,[34] lifestyle, cultural and ethnic differences, age, gender, marital status, hormonal effects, weather, cigarette smoking, consumption of alcohol, rest/tiredness, sleep, wearing/removing a prosthesis, occupation, leisure activities, sex, cognitive and emotional distractors, and other factors. This knowledge will become necessary in the postgenomic era, when identified polymorphic pain genes in humans and in relevant animal models[33–35] are studied for interaction with environmental variables (see Step 8 below).

Step 5: Assemble a Cohort of Pain-Phenotyped DNA Samples for the Study

Since there are currently no national or international repositories of DNA samples of orofacial pain patients (and of matched controls who underwent the same disease or event but did not develop chronic pain) for each orofacial pain syndrome,

there is a need to assemble a multicenter team to engage in collecting such samples (see Step 1). The pain clinicians and nurses in the team will contact potential participants, recruit them to the study, conduct an interview with the participants so they can complete the pain phenotyping questionnaires, draw a blood sample for DNA testing, and explain how to complete additional phenotyping questionnaires at home. Since chronic orofacial pain syndromes (eg, postherpetic neuralgia[1,4]) are more frequent in women and older people, it is strongly advisable to add these subjects to the cohort.

Epidemiologic geneticists and bioinformatic statisticians will be needed to assist in study design, in balancing out covariates by stratifying subjects, and in constructing the data set for segregation analyses against the genotypes. Most countries now have special ethics review boards to oversee genetic studies; these boards authorize the formation of a genetic bank for research purposes and monitor the study termination date, the date by which the material is to be discarded. Strict guidelines are imposed by such boards to keep the personal data of the participants confidential.

DNA for genetic studies is usually extracted from white blood cells. DNA from a venipunctured blood sample of 10 to 20 mL is enough for many hundreds of genotypings—a large but limited quantity. Since collecting a cohort of pain patients and their matched controls is so laborious and expensive, some research groups invest in "immortalizing" the DNA by introducing lymphoblasts to continuously produce the donor DNA. The lymphoblasts may also be used for mRNA expression analyses as a substitute for the patient's tissues.[36]

It is also advisable to keep the plasma of the blood sample and store it for future analyses of proteins, peptides, and cytokines, the levels of which may be affected by the genes one studies, and which can be indicators of changes occurring in the trigeminal system during chronic pain.

When a venipunctured blood sample is not available, a buccal smear or a drop of blood squirted from a pinpricked fingertip can be impregnated into a filter paper known as a "DNA card," dried, and stored.[37] Upon retrieval, the DNA card is chopped into small fragments, and each fragment is then amplified with as many "degenerative primers" as one can afford financially. This process can provide sufficient DNA for many hundreds of genotyping rounds and theoretically for as much as would be needed.

Step 6: Genotype the DNA Samples in Search of Polymorphic Pain Genes

There are 2 approaches to the identification of such genes: *(1)* a biased screen of candidate pain genes, and *(2)* an unbiased screen of the whole genome (a "genome-wide scan"), followed by a screen of candidate pain genes.

1. Screening Candidate Pain Genes. *Genotype Orthologous Genes Identified in Animal Pain Models.* Based on the hypothesis that chronic pain serves an important function that has a survival value that has led to its conservation in mammals, it is postulated that genes for chronic pain in human and animal models encode similar functions. It is estimated that 98% of the genes in the mouse have identical "orthologous" genes in humans.[38] Hundreds or thousands of genes are implicated directly and indirectly in mechanisms of chronic pain. Many of these are involved in orofacial pain, yet only a small fraction are polymorphic. While only these polymorphic genes can explain the variance in pain levels across individual inbred strains of animals and among humans, it is not known as yet whether pain genes that are polymorphic in rodents are also polymorphic in humans. This is testable, and in fact, can be tested in humans even before orofacial pain genes are identified in rodents. It is enough to know the chromosomal location of a putative orofacial pain gene in the mouse or rat to be able to identify whether this genetic locus also has a role in human orofacial pain. These chromosomal locations, termed quantitative trait loci, translate to known chromosomal regions in humans.

Based on the mouse-human chromosomal homology map,[39] it is possible to genotype the orthologous region in the human DNA cohort under study by the use of microsatellite genetic markers. An example of this approach is the authors' complementary project design to identify chronic orofacial pain genes in model animals. In this example a region on mouse chromosome 15 was identified as a quantitative trait locus for neuropathic pain following peripheral neurectomy. This chromosomal region corresponds to 2 human chromosomes, 1 on chromosome 8 and 1 on chromosome 22. In 650 DNA samples of leg amputees and mastectomized women these 2 regions were recently genotyped using several polymorphic microsatellite markers on each region. These markers can detect the presence of a nearby polymorphic gene from a distance of about 1 mega base pair (bp). The markers on chromosome 22, but not those on chromosome 8, were found to be

significantly associated with chronic pain levels. Microsatellite markers cannot identify a gene, but in this context, they were used to confirm that a region in human chromosome 22 harbors a gene relevant to chronic pain. Additional genotyping steps are then necessary to identify the gene itself. These steps use single nucleotide polymorphisms (SNPs) in candidate genes as markers for segregation with the pain trait. If a significant segregation is made using SNP markers, this is considered suggestive evidence for the identification of the target pain gene and for the existence of a polymorphic locus relevant for pain variance within 10 kbp of that SNP locus. An additional step of gene sequencing is then needed to identify the point mutation responsible for the pain trait.

SNPs currently reported in public databases have been mapped using only a few chromosomes. If no known SNP in a suspected gene differs between orofacial pain-affected and pain-free subjects in a studied cohort, a novel SNP in the gene that has not been described as of yet may still be "responsible" for the differing pain levels. This can readily be explored in the studied cohort. If no sequence mismatches in this gene are found to segregate with chronic pain levels, it is still possible that this gene is implicated in the variability of the pain trait under study by other mechanisms that involve gene expression levels. In this scenario, another gene affects splice variation and editing of the mRNA transcript of the pain gene, determining how many copies of the mRNA of this gene will be produced and what type of protein will be produced. Identification of the mechanism necessitates quantification of mRNA expression levels in neural structures where the target gene is expressed (eg, a nerve-end neuroma, the trigeminal ganglion, the brainstem, or brain structures).[40] Since this material is usually not available for research, animal models may serve as surrogates,[41] complemented if necessary by expression profiling of mRNA of lymphoblasts with immortalized DNA of chronic orofacial pain patients.

Screen Genes Whose Relevance for Orofacial Pain is Inferred from the Literature. Over the years, a large number of molecules have been identified as having a role in pain mechanisms, neural structural elements, chemicals that maintain membrane excitability and affect hyperexcitability, synaptic transmission (including neurotransmitters, modulatory neuropeptides, receptors, and synthesizing and catabolic enzymes), chemicals in various intracellular signal transduction pathways, and many others. The genes encoding some of these molecules have already been identified. Polymorphisms in some of these genes have been implicated in various normal neural functions and in neurologic and psychiatric abnormalities. A list prioritizing these candidate pain genes was published recently.[29]

There are currently a few laboratories that have running protocols for genotyping polymorphisms for some of these genes, and the number of such laboratories will surely increase in time. Their interest in a collaboration would be to find out whether genes for a pain syndrome they study have a role in orofacial pain as well. Showing that the same gene is shared between 2 or more pain diseases would have great basic and clinical importance for the development and use of drugs and diagnostic and prognostic kits having a broad indication.

2. Identifying all Chronic Orofacial Pain Genes in a Single Genome-wide Screen. The assumption that orofacial pain syndromes are complex inherited traits means that pain levels are determined by a number of genes having a relatively major effect on trait variance and an additional number of genes that modify their effect ("modifier genes"), each of which has a smaller effect on trait variance. Thus, the risk one inherits for these syndromes is likely determined by an ensemble of many genes. The steps described in this article are aimed at identifying genes with a relatively high impact on risk levels for a pain trait under study. These steps are biased by previous knowledge about the role certain candidate genes may have. However, to fully uncover the genetic and gene-by-environment components of the trait variance, one needs to identify *all* relevant genes, including the modifiers. It is expected that economically affordable microarray gene chips having a dense panel of about 500,000 SNP markers will soon become available. These chips will enable the identification of all polymorphic chronic pain genes an individual carries in a single run. There are numerous examples of genome-wide screens for various diseases. However, these are based on much smaller panels of genetic markers, hence their power to detect all polymorphisms affecting the trait is limited. See, for example, the findings of a recent study on multiple sclerosis.[42]

Step 7: Identify Mutations Affecting Orofacial Pain Levels in Various Ethnic Groups

It is quite likely that for any polymorphic orofacial pain gene there are ethnic-specific varieties of SNPs, introduced throughout the evolution of humans and these ethnic groups. This implies that meeting the treatment needs of peoples of various ethnic origins necessitates the identification of the SNPs affecting their susceptibility to developing chronic

orofacial pain by genetic screens of as many ethnic groups as possible. Multi-ethnic countries such as Canada are prime locations for such studies. These SNPs can then be used as markers for the predisposition of individuals to orofacial pain, as well as targets for novel drug development. An example is a recent study on autoimmunity genes.[43]

Step 8: Study Gene-by-Environment Interactions Affecting Chronic Orofacial Pain Levels

Several environmental risk factors have already been identified so far for chronic pain conditions in humans[11,31,32] and in animal models.[33–35] It is hoped that by the time orofacial pain genes are identified in humans, information on environmental variables affecting a particular pain will already have been collected (see Step 4). Syndromes being studied in humans should be modeled in rodents if this has not been done already. It could be of value to use molecular methods to induce in the mouse or rat the same mutations in the same genes as those affecting humans. By exposing these model animals to certain environmental variables that modulate pain levels in humans, one could study the effect of the environment on mRNA expression levels of the gene under study in various peripheral and central trigeminal neural structures involved in chronic orofacial pain.

Translational Pain Genetics: Gains Expected for Orofacial Pain Medicine

Some orofacial pain syndromes are difficult to diagnose.[1–4] New diagnostic kits to identify these syndromes will be developed based on genomic knowledge. Identifying which pain syndrome a patient has, and what other pain diseases this individual may be prone to developing, will help the clinician decide which therapy is suitable for an individual.

- The new genomic and phenomic knowledge could help reclassify orofacial pain syndromes based on genetic and environmental information. The new classifications would replace current definitions based on shared symptoms, for example, the poorly defined syndrome "atypical facial pain."[2]
- New prognostic kits will be developed to identify which treatment is best for an individual. These new kits could also provide means to select better subjects for clinical trials, thus minimizing costs of such trials and reducing the time new painkillers spend in testing.
- New pain-preventive medicine will be developed, for example, by individually tailoring drugs for effective "preemptive analgesia" or by providing better postoperative care.
- Novel orofacial pain mechanisms will be discovered by identifying genes and labeling their products. It is possible that previously unrecognized orofacial pain pathways will be discovered, as well as subcellular structures and mechanisms engaged in orofacial pain conditions. When novel cell types are identified, they will become targets for studies using electrophysiology, imaging and functional histological methods to unravel the mechanisms by which these pain syndromes are produced.
- Better animal models will be developed based on their relevance to human orofacial pain genes.
- Finally, in time it should be possible to apply gene therapy to replace "bad" genes with genes that do not "allow" an individual to develop chronic pain or to treat an individual who has already developed chronic orofacial pain.

Conclusions

Recent advances in the Human Genome Project have made it possible to benefit from the methodological developments in molecular genetics and epidemiological genetic statistics to promote orofacial pain medicine. The present article provides a practical guide on how to engage in a project on genetics and on environmental aspects of these unique pain diseases. A comparative approach that makes use of animal models of chronic orofacial pain and human models may provide knowledge that could revolutionize the preventive and palliative treatments of these pain types. This is the time for pain clinicians and researchers to become part of genetic research.

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