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Smoking, the xenobiotic pathway and clubfoot

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Abstract

Idiopathic clubfoot is a common orthopedic birth defect that affects approximately 135,000 newborns worldwide. It is characterized by equinus, varus and adductus deformities of the ankle and foot. While numerous studies suggest a multifactorial etiology, the specific genetic and environmental components have yet to be delineated. Maternal smoking during pregnancy is the only common environmental factor consistently shown to increase the risk for clubfoot. Moreover, a positive family history of clubfoot, in conjunction with maternal smoking, increases the risk twenty-fold. These findings suggest that genetic variation in smoking metabolism (xenobiotic) genes may increase susceptibility to clubfoot. Based on this reasoning, we interrogated eight candidate genes (*CYP1A1, CYP1A2, CYP1B1, CYP2A6, EPHX1, NAT2, GSTM1* and *GSTT1*), chosen based on their involvement in xenobiotic metabolism. Twenty-two SNPs and two null alleles in these genes were genotyped in a dataset composed of nonHispanic white and Hispanic multiplex and simplex families. Only rs1048943/*CYP1A1* had significantly altered transmission in the aggregate and multiplex NHW datasets (p=0.003 and p=0.009, respectively). Perturbation of *CYP1A1* can cause an increase in harmful, adduct forming metabolic intermediates. A significant interaction between *EPHX1* and *NAT2* was also found (p=0.007). Importantly, for *CYP1A2*, significant maternal (p=0.03; RR=1.24; 95% CI: 1.04–1.44) and fetal (p=0.01; RR=1.33; 95% CI: 1.13–1.54) genotypic effects were identified, suggesting that both maternal and fetal genotypes can negatively impact limb development. No association was found between maternal smoking status and variation in xenobiotic metabolism genes. Together, these results suggest that xenobiotic metabolism genes are unlikely to play a major role in clubfoot, however, perturbation of this pathway may still play a contributory role.

Keywords

clubfoot; smoking; tobacco; genetics; xenobiotic genes; CYP

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INTRODUCTION

Idiopathic talipes equinovarus, or isolated (non-syndromic) clubfoot is a common birth defect that has been recognized and described for centuries. Equinus, varus and adduction of the foot and ankle are the abnormal findings that comprise a clubfoot. In 50–75% of cases, the clubfoot is isolated, with no known cause(s) [Bakalis et al. 2002; Gurnett et al. 2008]. The birth prevalence of clubfoot is approximately 1/1,000 live births, although it can vary approximately 10-fold among different populations. The highest prevalence of clubfoot is in Polynesian populations (6.8/1,000 live births), while the lowest prevalence is observed in Orientals (0.57/1,000 live births) [Ching et al. 1969].

Although clubfoot is a common and well-studied birth defect, its cause(s) and risk factors have not yet been identified. Many etiologies of nonsyndromic clubfoot have been hypothesized and include vascular obstruction, abnormal muscle development, intrauterine growth restriction and neurological abnormalities [Dunn 1972; Hootnick et al. 1982; Reimann 1967; Turco 1981]. Evidence for a genetic etiology comes from twin and family studies. For example, the risk of clubfoot significantly increases as the number of affected relatives increases [Cardy et al. 2007; Cartlidge 1984; Engell et al. 2006; Harper 2004; Idelberger 1939; Lochmiller et al. 1998; Wynne-Davies 1964; Wynne-Davies 1965].

A number of additional environmental factors have been studied, but most have not been known to be consistently to be associated with clubfoot [Alderman et al. 1991; Byron-Scott et al. 2005; Moorthi et al. 2005; Wynne-Davies 1964]. While early amniocentesis (10–13 weeks) has been shown to increase the risk of clubfoot, maternal smoking is the only other environmental factor that has consistently been shown to be associated with clubfoot [Alderman et al. 1991; CEMAT 1998; Centini et al. 2003; Delisle and Wilson 1999; Skelly et al. 2002].

In the United States for 2007, the overall rate of cigarette smoking during pregnancy was 13.2%; for nonHispanic white (NHW) women the rate was more than six times then that of Hispanic women (18.1% versus 2.8%) [Heron et al.]. The relative risk for clubfoot when a mother smokes during pregnancy ranges from 1.34 (95% CI: 1.04–1.72) - 2.6 (95% CI: 1.6–4.0) suggesting an association between clubfoot and smoking (refs). The combination of smoking and a positive history of clubfoot increases the risk for clubfoot twenty-fold (Odds Ratio (OR) = 20.3 95% CI: 7.90–52.17) [Honein et al. 2000]. Therefore, certain genotypes (maternal and fetal) may confer an increased risk, which may be amplified by maternal smoking.

Cigarette smoke consists of more than 4,000 chemical compounds, including dioxins, dioxin-like compounds and other arylhydrocarbon receptor (AhR) agonists [Hecht 1999; Kitamura and Kasai 2007]. The main toxins in cigarette smoke are polycyclic aromatic hydrocarbons (PAHs) [Hecht 1999; Hoffmann and Hoffmann 1997]. Metabolism of tobacco smoke is accomplished through the xenobiotic metabolism pathway (Fig. 1 need to put in this fig which was 5) [Meyer 1996]. This pathway involves biotransformation of a xenobiotic compound by functionalization and/or conjugation reactions into polar, water-soluble metabolites that can be excreted [Meyer 1996; Voet 2004]. This pathway consists of phase I and phase II. Phase I is characterized by the functionalization reactions and utilizes enzymes capable of dehydrogenation/hydrogenation, oxidation, hydrolysis, reduction and mono-oxygenation [Meyer 1996]. Phase II consists of the conjugation reactions and utilizes enzymes capable of glucuronidation, sulphation, acetylation, GSH-conjugation and methylation.

Biotransformation through the xenobiotic metabolism pathway can detoxify a compound or it can create a more toxic/reactive intermediate metabolite. The damaging PAHs and

reactive metabolic intermediates from tobacco smoke cross the placenta and form adducts in maternal and fetal tissues [Czekaj et al. 2005; Gladen et al. 2000; Pasanen 1999; Shugart and Matsunami 1985]. Adducts are molecules that can covalently bind DNA or proteins and could therefore interfere with the normal developmental process by altering transcription and replication or damaging protein structure or function [Lopachin and Decaprio 2005; Meyer and Bechtold 1996; Uchida and Stadtman 2000]. For example, PAH-DNA adducts and dioxins from cigarette smoke are mutagenesis, carcinogenesis and teratogenesis [Kitamura and Kasai 2007; Nock et al. 2007].

Given the importance of the xenobiotic metabolism pathway in the clearance of harmful metabolic intermediates and the impact these intermediates may have on a developing fetus, the enzymes in this pathway may offer insight into the observed interaction between maternal smoking and family history on the risk for clubfoot. Variation within key genes involved in xenobiotic metabolism may lead to perturbation of the metabolism pathways and increase DNA or protein adduct forming compounds in fetal tissues which in turn could affect fetal development.

While many genes play a role in this pathway, only a few genes are known to specifically metabolize tobacco smoke. *CYP1A1*, *CYP1A2* and *CYP1B1* ? *CYP2A6*? are members of the cytochrome P450 (CYP450) superfamily and all play a role in phase I metabolism of compounds found in tobacco smoke, such as PAHs and dioxins [2004; Meyer 1996; Pavek and Dvorak 2008; Rodriguez-Antona and Ingelman-Sundberg 2006]. *CYP1A1*, *CYP1A2*, *CYP1B1* and *CYP2A6* expression has been identified in key organs, including the placenta [Pavek and Dvorak 2008]. Epoxide hydrolases (EH), such as *EPHX1*, also activate and detoxify PAHs during phase I [Omiecinski et al. 2000]. Specifically, EHs metabolize reactive epoxides to less-harmful dihydrodiol derivatives [Hassett et al. 1994b; Omiecinski et al. 2000]. Additionally, certain variants in *EPHX1* decrease its activity by 40%, inhibiting effective biotransformation of exogenous compounds [Hassett et al. 1994b].

Genes involved in phase II metabolism include *NAT2, GSTM1 and GSTT1. NAT2* is of particular interest because of its “slow acetylator” phenotype [Meyer and Zanger 1997]. The activity of the slow acetylation phenotype ranges from X-X of normal and is associated with increased in harmful adduct levels [Grant et al. 1990; Hecht et al. 2007; Meyer and Zanger 1997]. Approximately 40–70% of European and Northern American individuals have the “slow acetylator” phenotype [Meyer and Zanger 1997]. A study by Hecht, et al. (2007) suggested that the slow-acetylator phenotype of *NAT2* may play a role in the development of clubfoot. GST mu?μ (GSTM) and GST theta?τ (GSTT) are of interest because of their role in detoxification of activated nicotine metabolites and other xenobiotics during phase II [Pasanen 1999]. They are involved in the detoxification of epoxides created by the CYP450s [Bolt and Thier 2006]. Only about 40–60% of individuals in the population express *GSTM1* and, for those who do not express the gene, there is an increased susceptibility to DNA-adduct formation and cytogenetic damage [Seidegard and Pero 1985; Seidegard et al. 1986; Wiencke et al. 1990]. *GSTT1* also has a null allele that can be found in 10–40% of individuals [Seidegard et al. 1988].

These genes interact in the sense that they all play a role in a pathway responsible for metabolizing cigarette smoke, both through activating and detoxifying reactions. Because the level of genotoxic damage in individuals is the result of complex gene-environment and gene-gene interactions, genetic variants within multiple genes may act additively and alter the overall ability of the pathway to detoxify exogenous compounds or toxic intermediate metabolites [Georgiadis et al. 2004]. Specifically, individuals with polymorphisms that increase the activity in activating reactions and decrease the activity of inactivating reactions are more susceptible to the effects of genotoxic compounds [Georgiadis et al. 2004].

Therefore, a mutation in any one of these genes may have a large impact on the efficiency of biotransformation of toxic compounds and the presence of multiple mutations may increase the susceptibility even further. This study was undertaken to determine whether genetic variation in specific smoking xenobiotic genes involved in both phases I and II of cigarette smoke metabolism is associated with clubfoot in the presence or absence of maternal smoking.

MATERIALS AND METHODS

IRB Approval

This study was reviewed and approved by the Committee for the Protection of Human Subjects at the University of Texas Health Science Center at Houston (HSC-MS-09-0328) and all collaborating centers.

Clubfoot Study Samples and Sample Preparation

Probands and their families were identified at four pediatric orthopedic centers: Shriners Hospital for Children of Houston and Los Angeles, Texas Scottish Rite Hospital for Children of Dallas and University of British Columbia. The diagnosis of clubfoot was based on the presence of adducted forefoot, varus hindfoot and ankle equinus deformities as determined by either examination and/or by review of medical records. All patients with syndromic clubfoot were excluded.

All families were either NHW or Hispanic (Mexican) and ethnicity was self-reported. Family history and exposure information were obtained by interview with the proband's mother and/or by chart review. Two-generation pedigrees were constructed for all families and pedigrees were extended to include all affected individuals if a positive family history was elicted. Blood or saliva samples were collected on all available family members. DNA was extracted from the blood or saliva using either the Roche DNA Isolation Kit for Mammalian Blood (Roche, Basel, Switzerland) or the Oragene Purifier for saliva (DNA Genotek, INC., Kanata, Ontario, Canada) following the manufacturer's protocol.

The dataset consisted of 1,776 individuals from 619 families. Families were considered to be multiplex (242; 149 NHW and 92 Hispanic) or simplex (377 simplex; 149 NHW and 226 Hispanic) based on the presence or absence of a family history of clubfoot, respectively.

Gene and SNP Identification and Genotyping

Candidate genes were selected following a thorough literature review. Hundreds of enzymes play a role in xenobiotic metabolism, however only a subset of these enzymes are well-characterized and an even smaller subset have been shown to be specifically involved in the metabolism of tobacco smoke. Only genes known to metabolize compounds in cigarette smoke and that interact in multiple steps of a common general pathway were included in this study need some review refs here.

SNPs in the *CYP1A1*, *CYP1A2*, *CYP1B1*, *CYP2A6*, *EPHX1* genes were identified using NCBI and Ensembl websites. SNPs in the *NAT2* gene were identified previously [Hecht et al. 2007]. SNPs were selected based on a standard set of criteria including: heterozygosity >0.3, inter- and intragenic positions, coverage of the gene and tagging ability. SNPs with a higher heterozygosity that caused a missense mutation and/or tagged for multiple SNPs were always used. Table 1 lists the SNPs genotyped in this study. All SNPs were genotyped using TaqMan Genotyping Assays (Applied Biosystems, Foster City, CA) and detected on a 7900 HT Sequence Detection System (Applied Biosystems). All genotype results were entered into a Progeny and checked for incompatibility using Pedcheckref.

GSTM1 and *GSTT1* were genotyped to identify individuals with wild type or null alleles following the protocol of Arand *et al.* (1996). The *GSTM1* and *GSTT1* alleles were amplified simultaneously using Takara *Ex Taq* Polymerase PCR (Takara Bio USA). The PCR was modified by an addition of 0.5 μL of MgCl per reaction at an annealing temperature of 64°C. This method combines primers for *GSTM1*, *GSTT1* and *ALB* as an internal positive control into one assay [Arand et al. 1996]. Amplified samples were run on a 2% agarose gel and scored according to the presence of the wild type or null allele for both *GSTM1* and *GSTT1.* Absence of *GSTM1* or *GSTT1* were scored as null alleles [Arand et al. 1996].

Analysis

Allele frequencies and Hardy-Weinberg equilibrium (HWE) were calculated using SAS (v9.1). SNPs out of HWE (P<0.001) were excluded from the subsequent analyses. Chi-squared (X^2) analysis was performed to determine allele frequency differences between the NHW and Hispanic populations. Pair-wise linkage disequilibrium (LD) values (D' and r^2) were calculated using GOLD [Abecasis and Cookson 2000].

Linkage and/or association were tested using multiple analytic methods to extract the greatest amount of information from the data. Parametric and non-parametric linkage analyses were performed using Merlin [Abecasis et al. 2002]. Association was tested for using Pedigree Disequilibrium Test (PDT), genotype-pedigree disequilibrium test (geno-PDT) and Association in the Presence of Linkage (APL) [Martin et al. 2000]. [Martin et al. 2003] [Chung et al. 2006]. Two-SNP intrgenic haplotypes were evaluated using APL. Generalized estimating equations (GEE) as implemented in SAS was used to evaluate gene interactions [Hancock et al. 2007]. and Gene-environment interactions were assessed using FBATI hoffman ref.

Log-linear regression models were used to evaluate the independent effects of maternal and child genotypes [van Den Oord and Vermunt 2000]. Only one triad was selected per family. For each SNP, log-likelihoods were computed for the full models (including both maternal and child genotypes) and compared to the log-likelihoods computed for partial models (including either the maternal genotype or the child genotype only).

Chi-square analysis was used to evaluate the relationship between smoking and clubfoot in the presence of null *GSTM1* or *GSTT1* genotypes.

Protein function and transcription binding sites analyses

To evaluate the effect of the ancestral and alternate alleles on protein function, analyses of exonic missense mutations were performed using *in silico* SNPs3D and Polyphen programs [Teng et al. 2008; Yue et al. 2006]. Alibaba2, Patch and Transcription Element Search Software (TESS) were used to assess potential regulatory SNPs [Grabe 2002; Matys et al. 2006; Schug 2003]. The ancestral and alternate allele sequences were obtained from the NCBI Entrez SNP Database (www.ncbi.nlm.nih.gov).

RESULTS

Twenty-two SNPs in six genes, *CYP1A1, CYP1A2, CYP1B1, CYP2A6, EPHX1, NAT2,* and two null allele systems, *GSTM1* and *GSTT1*, were genotyped in our NHW and Hispanic families (Table 1). All SNPs had call rates of >95%. All SNPs were in HWE in the NHW group. In the Hispanic subset, three SNPS, rs1048943 (p=0.02), rs2470893 (p=0.0004) and rs4105144 (p=0.01), were not in HWE and were excluded from further. Allele frequencies for 16 of the 22 SNPs differed between the NHW and Hispanic groups; therefore the data was stratified by ethnicity. In addition, the data was further stratified by the presence or

absence of family history (FH) of clubfoot. There was signficant intragenic LD and the LD patterns were similar between ethnicities (data not shown).

Results of the single SNP analyses are presented in Table 2 and Supplemental Table 1. rs1048943 in *CYP1A1* had altered transmission in both the NHW aggregate and multiplex family subset (p=0.003 and p=0.009). SNPs in *CYP1A2* (1), *CYP1B1* (2), *EPHX1* (1) and NAT2 (1) had marginal evidence for altered transmission (Table 2A). None of the two-SNP haplotypes had altered transmission (data not shown).

For the Hispanic group, no SNPs had altered transmission in the aggregate group (Table 2B). rs7250713 in *CYP2A6* (p=0.01) and rs360063 in *EPHX1* (p=0.04) had altered transmission in the multiplex family subset, whereas rs1456432/*CYP1A1* (p=0.03) and rs360063/*EPHX1* (PDT: p=0.03; APL: p=0.01) had altered transmission in the simplex families. None of the two-SNP haplotypes had altered transmission.

Evidence for a gene interaction was seen only in the NHW group between rs105740 in *EPHX1* and rs1799929 in *NAT2* (p=0.007) (Table 3). Suggestive evidence for interactions was found for SNPs in *CYP2A6* and SNPs in *CYP1B1*, *EPHX1* and *NAT2*. There was minimal evidence for gene interactions in the Hispanic group.

Evidence for the independent effects of maternal and fetal genotypes was found for only two SNPs in CYP1A2 (Table 4). For rs11854147, a significant maternal genotypic effect (p= 0.03) was found with a relative risk of 1.24 (95% CI: 1.04–1.44). A significant fetal genotypic effect (p= 0.01), with a relative risk of 1.33 (95% CI: 1.13–1.54), was found for rs2470890‥

Two associated SNPs, rs2472299/*CYP1A2* and rs4105144/*CYP2A6* are located potential regulatory regions (Table 5). All three DNA-binding site algorithms predicted that the alternate rs2472299 allele eliminates a glucocorticoid receptor (GR) TFBS. In contrast, DNA binding sites for rs4105144 in *CYP2A6* were inconsistent although two programs predicted a change between ancestral and alternate alleles.

Forty-seven percent of individuals genotyped were homozygous for the *GSTM1* null allele and 18% for *GSTT1* null allele, which is consistent with previous reports [Seidegard et al. 1988]. Smoking was reported by 23% of NHW mothers and 4.6% of Hispanic mothers. There was no evidence for an association between *in utero* exposure to maternal smoking and *GSTM1* or *GSTT1* maternal or fetal phenotypes. In addition, there was no difference in the percentage of null mothers versus null fathers for either gene for either ethnic group. Lastly, FBAT-I did detect any evidence for an effect of smoking in any of SNPs in the other six genes.

DISCUSSION

An explanation for the development of clubfoot has been sought almost since the beginning of recorded history. The current hypothesis invokes both genes and environmental factors, although the exact roles of each still need to be defined refs. Candidate gene studies have begun to uncover the clubfoot etiologic pathways. For example, apoptotic genes, such as Casp8/10 and Casp3, as well as HOX genes have recently been associated with clubfoot heck[Ester et al. 2007; Ester et al. 2009]. Maternal smoking is the only common environmental exposure that has consistently been shown to increase the risk of clubfoot [Alderman et al. 1991; Honein et al. 2000; Skelly et al. 2002]. Importantly, the risk of clubfoot is significantly increased for women who smoke during pregnancy and have a positive family history [Honein et al. 2000]. These observations led us to hypothesize that genetic variation in smoking metabolism genes increases susceptibility to clubfoot.

Therefore, we interrogated eight candidate genes, chosen for their involvement in cigarette smoke metabolism [Ambrosone et al. 1996; Omiecinski et al. 2000; Rodriguez-Antona and Ingelman-Sundberg 2006][Pasanen 1999; Seidegard et al. 1988].

We hypothesized that perturbation of phase I reactions of xenobiotic metabolism, that increase enzyme activity and adduct formation, play a role in the development of clubfoot. Compounds from cigarette smoke form DNA and protein adducts, which can cause mutagenesis and teratogenesis [Detmar et al. 2008; Kitamura and Kasai 2007; Nock et al. 2007]. Additionally, simultaneous increase in phase I activity with a perturbation in phase II activity may also play a role. Because phase I xenobiotic metabolism genes create harmful metabolic intermediates in the normal biotransformation pathway, it may be that an increase in phase I enzyme activity and a decrease in phase II degradation of these intermediates increases the concentration of harmful compounds to damaging levels that can interfere with fetal development. Some of the metabolic intermediates known to be produced by phase I xenobiotic metabolism are active oxygen species and DNA or protein binding adducts [Meyer 1996]. While it has been suggested that harmful oxygen species and adducts can interfere with normal fetal development, the role of these compounds in the pathogenesis of clubfoot is unknown [Izzotti et al. 2003; Pasanen 1999; Pinorini-Godly and Myers 1996]. Our results suggest that an increase in harmful metabolic intermediates could contribute to abnormal foot development or rotation of the foot.

Considering the strength of the association between clubfoot and smoking from previous population-based studies, there was surprisingly minimal evidence to support a role of these eight genes in clubfoot. The strongest evidence for association was for *CYP1A1* (rs1048943; p=0.003) in the NHW dataset in the single SNP analysis. The is a missense mutation (1384A>G) in exon 7 that changes an isoleucine to a valine at amino acid position 462 (www.ncbi.nlm.nih.gov/) and confers higher phase I enzymatic activity, which may increase exposure to harmful, adduct forming, metabolic intermediates [Lamba et al. 2002; Schwarz et al. 2005]. Another *CYP1A1*-related SNP, rs1456432, gave minimal evidence of altered transmission in the Hispanic group. This SNP is located 9.1 kb downstream of *CYP1A1* and therefore, may play a role in stabilization of the mRNA [Wickens et al. 2002].

One other significant finding in the NHW group was the interaction between rs1051740 in *EPHX1* and rs1799929 in *NAT2* (p=0.007) (Table 3). *EPHX1* plays major role in hydrolysis of PAH, a phase I process [Hassett et al. 1994a; Omiecinski et al. 2000]. The rs1051740 variant has decreased activity and has been associated with decreased formation of DNA adducts [Georgiadis et al. 2004; Nock et al. 2007]. *NAT2* plays an important role in phase II reactions of tobacco smoke metabolism [Ambrosone et al. 1996]. The rs1799929/*NAT2* codes for a synonymous amino acid change in exon 2, which could decrease the rate of *NAT2* translation [Nielsen et al. 2007]. Importantly, *NAT2* is known to have variants with decreased activity, though the *in vivo* effect of the rs1799929 on enzyme activity is not known [Meyer and Zanger 1997].

Our results also identified other marginal associations that could potentially be important. Many of these SNPs are known or predicted to alter the function of the gene and may impact the efficiency of the xenobiotic metabolism pathway by perturbing the activity of phase I and/or phase II reactions. While these associations are marginal, they suggest that multiple variants are required to perturb different parts of the xenobiotic metabolism pathway, and thereby contribute to clubfoot. Additional studies are needed to further explore these potential interactions and confirm the associations.

One such association involves rs1799931 in *NAT2*, which had marginal evidence of association in the single SNP analyses only in NHW simplex cases. This SNP encodes a

missense mutation that changes a glycine to a glutamine in the mature protein (www.ncbi.nlm.nih.gov/snp/) and is predicted to affect protein stability (SNPs3D.org). I*n vitro* studies showing decreased activity in Chinese hamster ovary (CHO) cells and unstable protein formation in E. coli [Deguchi 1992; Hein et al. 1994; Meyer and Zanger 1997]. The activity of this SNP *in vivo* is not known. These results support our earlier finding of suggestive evidence for an association with rs1799931 in both NHW and Hispanic simplex families Hecht, et al., 2007. However, in that study, a more significant association was found for the Hispanic simplex families suggesting that this variant may be a greater risk factor in the Hispanic population. The current results suggest that variation in *NAT2* may also be an important risk factor for the NHW population.

In addition to the significant interaction between rs1799929/*EPHX1* and rs105740/*NAT2*, a marginal interaction was found between rs1051740 in *EPHX1* and rs1801280 in *NAT2*, the latter of which causes a “slow acetylator” phenotype [Bell et al. 1993; Cascorbi et al. 1995; Meyer and Zanger 1997]. Interestingly, we observed a marginal interaction between rs1056836 in *CYP1B1* and the same *NAT2* SNPs seen in the interactions with *EPHX1* (rs1799929 and rs1801280). *EPHX1* and *CYP1B1* interact through sequential reactions in phase I metabolism of PAH and *NAT2* is a well-characterized phase II enzyme with variants that have been shown to cause an increase in harmful adduct levels [Grant et al. 1990; Meyer and Zanger 1997; Nock et al. 2007]. These results provide additional support for the findings by Hecht, et al., 2007 and provide additional evidence that *NAT2* plays a role in clubfoot. Moreover, these results support our hypothesis that perturbation of phase I and phase II enzymatic activity may leads to an increased risk of clubfoot.

Other marginal but potentially interesting interactions involved *CYP1B1, CYP2A6* and *EPHX1*. Because almost all of the marginal gene interactions involved one of these three genes, our results suggest that *EPHX1* and *CYP2A6* may play a role in clubfoot in the NHW group while *CYP1B1* may be important in Hispanics. Additionally, the gene interaction results are intriguing when the functional effect of the SNPs and the possible effect on the metabolic pathway are considered. In general, we found interactions involving between variants that are known to alter the activity of the phase I enzyme or could affect regulation of the gene. Although the these findings need to be further explored, collectively these results support our hypothesis that disruption of normal activity in both phase I and II of xenobotic metabolism can increase the concentration of harmful intermediates and may play a role in clubfoot.

GSTM1 and *GSTT1* also play a role in phase II of tobacco smoke metabolism and the null alleles of both genes cause absence of enzymatic activity [Pasanen 1999]. Loss of activity causes increased susceptibility to DNA adducts and could increase harmful metabolic intermediates [Pemble et al. 1994; Seidegard and Pero 1985; Seidegard et al. 1986; Seidegard et al. 1988; Wienckeet et al. 1990]. Interestingly, our results provide no support for a relationship between a homozygous null allele at either locus and an increased risk of clubfoot, even in the presence of smoking. However, these results must be carefully interpreted because heterozygotes cannot be discriminated from homozygous wild type individuals [Pemble et al. 1994; Seidegard et al. 1988]. Our results suggest that *GSTM1* and *GSTT1* null genotypes, whether they are present in the fetus or the mother, are not associated with an increased risk of clubfoot when the mother smokes during pregnancy.

Because both the maternal and fetal smoking metabolism genes may affect the risk for clubfoot, we evaluated whether each SNP possessed an independent maternal genetic effect or an independent inherited genetic effect in the fetus [van Den Oord and Vermunt 2000]. For *CYP1A2*, we found evidence for a significant deleterious effect for rs11854147 (p=0.03; RR=1.24; 95% CI: 1.04–1.44) in the mother and for rs2470890 (p=0.01; RR=1.33; 95% CI:

1.13–1.54) when in the fetus. This suggests that variation in CYP1A2 can increase the risk of clubfoot but the maternal or fetal mechanisms differ. Differences in the gene expression, induction and enzyme activity between maternal and fetal tobacco metabolism genes have been reported and these results support evidence that variants in tobacco metabolism genes have different consequences when they are of maternal and/or fetal origin [Legraverend et al. 1984; Pasanen 1999; Pavek and Dvorak 2008].

Interestingly, although an association between xenobiotic metabolism genes and clubfoot was found, we were unable to confirm the association between maternal smoking and clubfoot reported in other studies [Alderman et al. 1991; Dickinson et al. 2008; Honein et al. 2000; Skelly et al. 2002]. In our dataset, only 23% of NHW mothers and 4.6% of Hispanics mothers reported smoking during pregnancy as compared to 18.1% and 2.8%, respectively in the general population. In epidemiological studies that have found an association between maternal smoking and clubfoot, an average of 30% of mothers reported smoking during pregnancy [Dickinson et al. 2008; Honein et al. 2000; Skelly et al. 2002]. ?do any of these studies have a breakdown by ethnicity?? Because smoking does not appear to be as great a risk factor in our population, we would not expect to see strong associations between xenobiotic metabolism genes and clubfoot. Evaluation of the role of these genes in a dataset for which smoking is found to significantly increase the risk of clubfoot may reveal stronger associations between these genes and clubfoot and should be explored in future studies.

If tobacco smoke exposure is not affecting the fetus through the xenobiotic metabolism pathway, there may be other etiologic mechanisms. For example, early amniocentesis also increases the risk of clubfoot suggesting that a teratogenic mechanism may be common to both exposures. The simplest explanation would be vascular insufficiency and hypoxia that would deprive the fetus of blood flow and necessary nutrients. Maternal smoking and nicotine exposure in mice specifically reduces blood flow and increases vascular resistance in the uterus [Albuquerque et al. 2004; Bruner and Forouzan 1991; Clark and Irion 1992; Detmar et al. 2008; Shea and Steiner 2008]. Moreover, mice exposed to PAHs have been shown to have abnormal vasculature in the placenta that significantly reduces arterial surface area and volume of the fetal arterial vasculature [Detmar et al. 2008]. Reduction in vascular efficiency and an increased susceptibility to hypoxia could cause abnormal limb development and has been shown to cause transverse limb defects in prolonged (30–60 minutes) cases of anoxia [Webster and Abela 2007]. Therefore, variable degrees of hypoxia may increase the risk of other limb abnormalities, the mildest of which could be clubfoot.

The results of this study are important but must be carefully interpreted until validation studies can be undertaken. We focused on only eight of the most important tobacco smoke metabolism enzymes. There are likely hundreds of genes involved in the overall biotransformation of the compounds found in tobacco smoke, which were not considered in this study. While the genes in this study are the most likely candidates, we cannot rule out that other genes play a major role or interact with the genes in this study and contribute to the clubfoot phenotype. Our results suggest that there is an association between *CYP1A1* and an interaction between *EPHX1* and *NAT2* xenobiotic metabolism genes and an increased risk for clubfoot. The genes in this study are likely to interact in pathways that affect fetal development. Further studies are needed to better delineate the role of maternal smoking and xenobiotic metabolism genes during pregnancy and the effects on the developing fetus.

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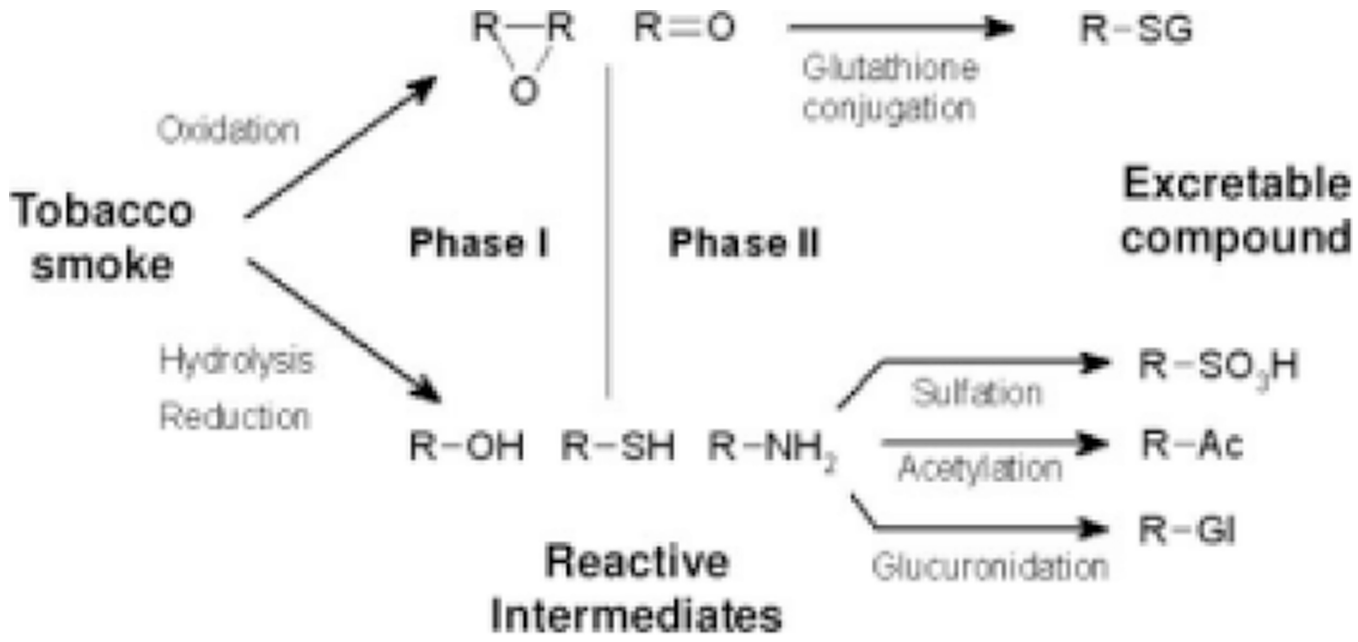

Figure 1.
Xenobiotic metabolism pathway. Adapted from Tim Vickers, http://en.wikipedia.org/wiki/File:Xenobiotic_metabolism.png.

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Table 1

Smoking Metabolism Genes: SNP Location, Alleles and Ethnic Frequencies[a]

Xenobiotic phase	Gene	SNP	Base pair no.	Alleles[b]	Location[c]	Type[d]	cDNA change	Protein change	MAF	HCF[e]
I	*CYP1A1* 15q22–24 5.99kb	rs2470893	72806502	G/A	U	–	–	–	0.30	0.13
		rs1048943	72800038	A/G	E	M	1384A>G	Ile462Val	0.04	0.35
		rs1456432	72790104	G/A	D	–	–	–	0.16	0.44
	CYP1A2 15q22-qter 7.76kb	rs2472299	72820453	G/A	U	–	–	–	0.28	0.27
		rs2470890	72834479	C/T	E7	S	1548T>C	Asn516Asn	0.37	0.68
		rs11854147	72839824	C/T	D	–	–	–	0.33	0.61
	CYP1B1 2p22-p21 8.55kb	rs4646429	38160439	A/G	U	–	–	–	0.32	0.31
		rs10012	38155894	G/C	E2	M	142C>G	Arg48Gly	0.32	0.33
		rs1056836	38151707	G/C	E3	M	1294C>G	Leu432Val	0.42	0.25
		rs163084	38144420	T/C	D	–	–	–	0.20	0.14
	CYP2A6 19q13.2 6.90kb	rs4105144	46050464	C/T	U	–	–	–	0.32	0.25
		rs7250713	46047035	C/G	I2	–	–	–	0.40	0.34
		rs7246742	46037235	G/T	D	–	–	–	0.13	0.18
	EPHX1 1q42.1 20.29kb	rs2854450	224079200	C/T	U	–	–	–	0.20	0.18
		rs1051740	224086256	T/C	E3	M	337T>C	Tyr113His	0.30	0.41
		rs2234922	224093029	A/G	E4	M	416A>G	His139Arg	0.17	0.08
		rs360063	224102932	G/A	D	–	–	–	0.44	0.48
II	*NAT2*8p23.1-p21.3 9.97kb	rs1041983	18302075	C/T	E2	S	282C>T	Tyr94Tyr	0.33	0.31
		rs1801280	18302134	T/C	E2	M	341T>C	Ile114Thr	0.44	0.32
		rs1799929	18302274	C/T	E2	S	481C>T	Leu161Leu	0.42	0.32
		rs1799930	18302383	G/A	E2	M	590G>A	Arg197Gln	0.30	0.18
		rs1799931	18302650	G/A	E2	M	857G>A	Gly286Glu	0.04	0.13
	GSTM1			WT/null	–	Null	–	Null	0.47	0.56
	GSTT1			WT/null	–	Null	–	Null	0.20	0.13

[a]HCF = Hispanic corresponding frequency to NHW minor allele; MAF = minor allele frequency in non-Hispanic white sample; WT = wild-type.

[b]Ancestral allele/alternate allele.

[c]U = upstream, E = exon, I = intron, D = downstream.

[d] Type of mutation: M = missense; S = synonymous.

[e] Values in bold = HCF significantly different from MAF [$p < 0.01$].

Table 2

Single SNP Association Results by Family History[a]

A. NHW

		All			Multiplex			Simplex		
Gene	dbSNP	PDT	GENO-PDT	APL	PDT	GENO-PDT	APL	PDT	GENO-PDT	APL
CYP1A1	rs1048943	0.003	0.003	0.12	0.009	0.009	0.71	0.17	0.17	0.06
CYP1A2	rs2472299	0.85	0.29	0.03	0.61	0.22	0.27	0.21	0.43	0.07
CYP1B1	rs1056836	0.53	0.76	0.14	0.92	0.93	0.81	0.13	0.14	0.05
CYP1B1	rs163084	0.10	0.14	0.05	0.40	0.59	0.40	0.05	0.04	0.05
EPHX1	rs2234922	0.05	0.09	0.13	0.05	0.06	0.06	0.73	0.68	0.93
NAT2	rs1799931	0.12	0.07	0.55	0.59	0.70	0.41	0.01	0.01	0.04

B. Hispanic

		All			Multiplex			Simplex		
Gene	dbSNP	PDT	GENO-PDT	APL	PDT	GENO-PDT	APL	PDT	GENO-PDT	APL
CYP1A1	rs1456432	0.44	0.68	0.52	0.39	0.70	0.37	0.03	0.12	0.21
CYP2A6	rs7250713	0.37	0.17	0.74	0.88	0.39	0.01	0.25	0.20	0.34
EPHX1	rs360063	0.62	0.13	0.28	0.42	0.08	0.04	0.03	0.15	0.01

[a]Results for $p \leq 0.05$.

Table 3

Gene-Gene Interactions

A. NHW				
Gene	SNP	Gene	SNP	*p* value
EPHX1	rs1051740	*NAT2*	rs1799929	0.007
		NAT2	rs1801280	0.03
		CYP2A6	rs7250713	0.05
	rs360063	*CYP2A6*	rs4105144	0.04
	rs2234922	*CYP2A6*	rs7246742	0.05
CYP2A6	rs4105144	*CYP1B1*	rs1056836	0.02
		NAT2	rs1799930	0.04
B. Hispanic				
Gene	SNP	Gene	SNP	*p* value
CYP1B1	rs1056836	*NAT2*	rs1799929	0.04
			rs1801280	0.04
	rs163084		rs1799929	0.05
	rs1056836	*CYP1A2*	rs2472299	0.05
CYP1A1	rs1456432	*CYP1A2*	rs2472299	0.04

Table 4

Log-Linear Regression Modeling of Maternal and Fetal Genotypes[a]

		RR (95% CI)		LRT *p*-value	
Gene	**SNP**	**Fetal**	**Maternal**	**Fetal**	**Maternal**
CYP1A2	rs2470890	1.33 (1.13–1.54)	1.23 (1.02–1.45)	0.01	0.06
	rs11854147	1.19 (0.99–1.39)	1.24 (1.04–1.44)	0.99	0.03

[a]RR = relative risk; LRT = log likehood ratio test.

Table 5

Predicted Transcription Factor Binding Sites for 5′-Associated SNPs[a]

		Alibaba2 Alleles		Patch Alleles		TESS Alleles	
Gene/SNP	**Location**	**Ancestral**	**Alternate**	**Ancestral**	**Alternate**	**Ancestral**	**Alternate**
*CYP1A2/*rs2472299	8.7 kb upstream	GR	None	GR, AR	None	GR, AR	None
*CYP2A6/*rs4105144	2.3 kb upstream	None	PU.1	None	None	Bcd, Ft2.2	LEF

[a]AR = androgen receptor; Bcd = bicoid; GR = glucocorticoid receptor; LEF = lymphoid enhancer factors.

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Table 6

Functional Effects for Smoking Metabolism Gene Interactions[a]

A Interactions between Phase I Genes

Gene 1	SNP 1	Functional effect	Gene 2	SNP 2	Functional effect	*p* value	Pop.
CYP1B1	rs1056836	Variant has increased metabolism of BaP and decreased metabolism of BaP-7,8-dihydrodiols; increased risk for adducts[b–c]	*CYP2A6*	rs4105144	5′ of gene; may affect TFBS[e]	0.02	NHW
			CYP1A2	rs2472299	5′ of gene; may affect TFBS[e]	0.06	H
CYP1A1	rs1456432	3′ of gene;possible enhancer region	*CYP1A2*	rs2472299	5′ of gene; may affect TFBS[e]	0.04	H
EPHX1	rs360063	3′ of gene;possible enhancer region	*CYP2A6*	rs4105144	5′ of gene; may affect TFBS[e]	0.04	NHW
	rs2234922	Variant has decreased activity and decreased adducts[b]	*CYP2A6*	rs7246742	3′ of gene; possible enhancer region	0.06	NHW
	rs1061740	Variant has decreased activity and decreased adducts[b]		rs7250713	Intronic	0.06	NHW

B. Interactions between Phase I and Phase II Genes

Phase I gene	SNP	Functional effect	Phase II gene	SNP 2	Functional effect	*p* value	Pop.
EPHX1	rs1061740	Variant has decreased activity and decreased adducts[b]	NAT2	rs1799929	Activity unknown in vivo[d]	0.007	NHW
			NAT2	rs1801280	Decreased activity = “slow acetylator”[d]	0.03	NHW
CYP2A6	rs4106144	5′ of gene; may effect TFBS[e]	*NAT2*	rs1799930	Decreased activity in vitro[d]	*NAT2*	NHW
CYP1B1	rs1056836	Variant has increased metabolism of BaP and decreased metabolism of BaP-7,8-dihydrodiols; increased risk for adducts[b–c]	*NAT2*	rs1799929	Activity unknown in vivo[d]	0.04	H
			NAT2	rs1801280	Decreased activity = “slow acetylator”[d]	0.04	H
	rs163084	3′ of gene; possible enhancer region	*NAT2*	rs1799929	Activity unknown in vovo[d]	0.05	H

[a] Published literature listed in References. TFBS = transcription factor binding site; variant = alternate allele.

[b] Nock et al, 2007.

[c] Georgiadis et al, 2004.

[d] Meyer and Zanger, 1997.

[e] AliBaba2,Patch, TESS.