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A MEDICAL MOLECULAR GENETICS OF OROFACIAL CLEFTING: ROLE OF ABC TRANSPORTER POLYMORPHISMS IN DISEASE RISK

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Dedicated to my mom and dad

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SUMMARY

Non-syndromic oral clefts are considered ‘complex’ or ‘multifactorial’ in that both genes and environmental factors contribute to the etiology. Thus far, several genes such as *TGFA*, *MSX1*, *TGFB3*, *D4S192*, *RARA*, *MTHFR*, *RFC1*, *GABRB3*, *PVRL1*, and *IRF6* were found to be associated with oral clefting. However, environmental factors such as maternal smoking or drug treatment in pregnancy especially during the early stage of fetal development can influence the disease risk. Thus, barrier organs in mother and fetus play crucial role in controlling the fetal exposure to potentially toxic xenobiotics. ATP-binding cassette (ABC) proteins are active efflux transporters regulates the traffic of xenobiotics across cells in maternal barrier organs and placenta as a fetal barrier organ.

Family based association study and case-control study were conducted to investigate the role of potentially functional polymorphisms within four ABC transporter genes, namely ***ABCB1*** (e12/C1236T, e21/G2677T/A, e26/C3435T), ***ABCC1*** (5'FR/G-260C), ***ABCC2*** (e1/C-24T, e10/G1249A, e25/G3542T), and ***ABCG2*** (e2/G34A, i9/T-357C, e5/C421A) in susceptibility to non-syndromic orofacial clefting. For the family based study, 150 nuclear families of single affected offspring with oral clefs were recruited from Singapore and Taiwan. The phenotype of interest included all forms of non-syndromic orofacial clefts including cleft lip (unilateral or bilateral) with or without cleft palate (CL/P), cleft palate, or both. In the recruited families, parents were all unaffected with oral clefts.

In the case-control study, the affected member from the recruited family will represent the cases while 189 healthy Chinese mothers were recruited to form the control group.

The SNP genotype data of 128 oral cleft Chinese children as well as 129 Chinese mothers with oral cleft proband were separately compared against the control group.

Genomic DNAs extracted from the families' peripheral blood and the controls' cord blood samples were genotyped for the target SNPs through multiplex PCR, multiplex minisequencing, sequencing, and TaqMan® SNP Genotyping Assay.

An extended transmission disequilibrium analysis on the genotype data of the families revealed that only SNPs within the *ABCB1* gene (e12/1236, e21/2677, and e26/3435), but not SNPs in other ABC genes were significantly associated with orofacial clefting ($P<0.05$). Interestingly, the results of the family based study were validated by the case-control analysis, which showed significant association for the SNP C1236T even after correction for multiple testing in children with oral clefts using Fisher's exact test. However, there was no significant difference in the SNPs allele distribution between the mothers of the oral clefts and the controls, suggesting the fetal *ABCB1* genotype not the maternal genotype is crucial in the disease risk. Moreover, in 129 Chinese families with oral cleft offspring, no significant difference was observed when proportions of the homozygote maternal genotypes were compared with the paternal genotypes in the presence of a homozygote proband for the target SNPs, suggesting no synergistic effect for the maternal genotype on fetal genotype in the disease vulnerability.

The findings of this study can favor the important role of the placental ABCB1 in protecting the fetus at the time when lip and palate are forming and suggest that fetal *ABCB1* polymorphisms can influence susceptibility to orofacial clefting.

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CONFERENCE PRESENTATION

A polymorphism at the *MDR*1 gene locus is significantly associated with non-syndromic oral clefting in family-based association study.

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1 INTRODUCTION

Placenta is the organ that brings into close apposition the blood circulations of two human beings, mother and fetus for exchanging of nutrients and gases between mother and fetus and removing fetal waste products and xenobiotics including drugs. The human placenta is formed by both fetal (chorionic plate and chorionic villi) and maternal tissues (decidua basalis). The placenta consists of syncytiotrophoblast and cytotrophoblast (or Langerhans) layers. The apical membrane of the syncytiotrophoblast is directly bathed in the maternal blood while the basolateral surface is in contact with either the discontinuous cytotrophoblast layer, with stromal tissue or with fetal blood vessels. Thus, the passage of toxins or nutrients from maternal to fetal circulation requires translocation across the brush border (apical) and basolateral membrane of the syncytiotrophoblast, as well as the continuous endothelium of the fetal capillaries. Transporter proteins, such as ATP-binding cassette transporters (ABC-transporter) are located at apical and basolateral surfaces of syncytiotrophoblast and endothelial cells of fetal capillaries (Figure 1-1). These proteins are able to efflux environmental toxicants or drugs ingested by the mother to the maternal circulation. Therefore, they are supposed to be a functional part of the placental barrier and the expression of transporter proteins within these structures is important in protecting the fetus from toxic xenobiotics. Because the trophoblast cells forming the materno-fetal barrier are of fetal origin, expression of transporter proteins in placental is determined by the fetal genotype. Hence, placenta is considered the first fetal organ exposed to exogenous substances including drugs in the maternal circulation.

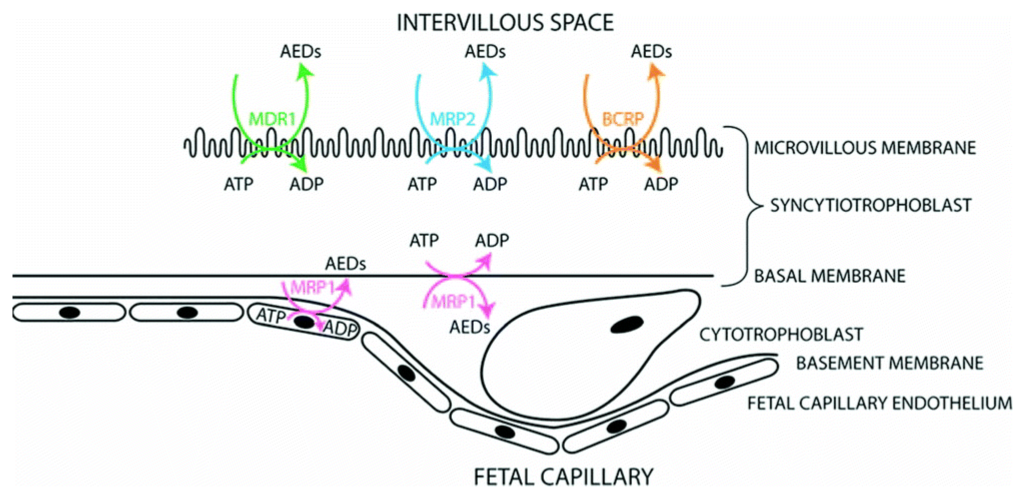

Figure 1-1 Schematic of placental localization and function of MDRPs. ATP hydrolysis drives the efflux of Antiepileptic drugs (Atkinson et al. 2007) [Reprinted, with permission].

Functional genetic polymorphisms in major ABC-transporter genes expressed in the placenta may impair the detoxification role of these proteins in protecting the fetus from feto-toxic substances, thus increasing the risk of complex genetic disorders mediated by xenobiotics exposure. In the current study, we aimed to search the association between functional polymorphisms in four members of ABC-transporter family, ABCB1; ABCC1; ABCC2; ABCG2, with orofacial clefting in which both genes and environmental factors influence the disease risk.

Orofacial clefts (i.e., cleft lip [CL], cleft lip and palate [CLP], or cleft palate [CP] alone) are one of the most common birth defects (approximately one case in every 500-550 births) and varies widely in severity. Pathologically, orofacial clefts are divided into syndromic ,babies having other birth defects, and nonsyndromic.

In normal development, facial morphogenesis begins with migrating of neural crest cells into the facial region. The upper lip is derived from medial nasal and maxillary

processes and in the normal situation; the processes grow into an open space by means of migration and multiplication of neural crest cells. The formation of a baby's lip will close by about 5 to 6 weeks and the palate will close by about 10 weeks after gestation. The cleft may happen because of reducing migration, multiplication, or both of neural crest cells, thus preventing merging between the medial nasal and maxillary processes around 5 weeks' gestation, or it can be because of killing some cells that are already in that location on one or both sides.

However, the cleft may affect only the upper lip, or it may more deeply extend into the maxilla and the primary palate. Generally, a cleft lip/palate occurs more often than the isolated cleft palate.

Being a complex disorder, orofacial malformations can be influenced by both individual and environmental factors such as:

1. Race: Asians and some groups of native Americans are appeared to be more affected with cleft lip/palate, but the risk for isolated cleft palate appears similar in all racial groups.

2. Gender: cleft lip/palate occurs more frequently in males, but isolated cleft palate occurs more frequently in females.

3. Genetics: studies have revealed association between nonsyndromic orofacial clefts and a number of genes, such as *TGFA, MSX1, TGFB3, D4S192, RARA, MTHFR, RFC1, GABRB3, PVRL1, and IRF6.* However, causal genes may interact with one another and/or with environmental risk factors.

4. Mother's health: maternal illness or infection as well as a deficiency of folic acid may be liable for these anomalies. Studies also suggest a link between development of cleft lip/ palate and **maternal medication** (such as antiepileptic drugs), **maternal smoking**, and **alcohol** use.

However, a cleft lip/palate develops when exogenous factors, such as toxic drugs, interact with a genetically susceptible genotype, during the early stage of development (i.e., 5 or 10 weeks after gestation), which is a critical period for fusing the processes and the palate.

1.1 ATP Binding Cassette Transporter

ATP-binding cassette transporters (ABC-transporter) are members of a largest protein superfamily in both prokaryotic and eukaryotic organisms. Currently, more than 49 members of this super-family have been identified in human, which are classified into seven distinct subfamilies based on the organization of their domains and amino acid homology. The subfamily designations are as follows: ABCA, ABCB, ABCC, ABCD, ABCE, ABCF, and ABCG. Members of the ABCB subfamily can be referred to as the “MDR-ABC transporters” and members of ABCC subfamily can be referred to as the “MRP-ABC transporters”.

These transmembrane proteins utilize the energy of adenosine triphosphate (ATP) hydrolysis for the translocation of various substrates, including metabolic products, lipids and sterols, and drugs across extra- and intracellular membranes. Given their vital role, human ABC transporters are medically important. They are found to be involved in several Mendelian diseases and complex genetic disorders, including cystic fibrosis, neurological disease, immune deficiencies, retinal degeneration, cholesterol and bile transport defects (Dubin-Johnson syndrome), Tangier disease, anemia, and drug response phenotypes that arise from polymorphisms in ABC genes and rarely due to complete loss of function of single ABC proteins (Pohl et al. 2005). ABC transporters are also found to be responsible for multiple drug resistance (MDR) against a variety of chemical compounds, such as anti-cancers, anti-arrhythmics, anti-

depressants, anti-pyschotics, anti-viral drugs, and other drugs. Studies have revealed that these proteins are overexpressed in tumor cells, thus conferring them resistance to chemotherapy. Table 1 shows a partial list of drugs transported by these proteins.

1.1.1 Structure and Function

All ABC transporters consist of two distinct domains, the hydrophobic transmembrane domain (TMD) and the hydrophilic nucleotide-binding domain (NBD). The TMD, also known as membrane-spanning domain (MSD) or integral membrane (IM) domain embedded in the membrane bilayer. It recognizes a variety of substrates and undergoes conformational changes to transport the substrate across the membrane. The sequence and architecture of TMDs is variable, reflecting the chemical diversity of substrates that can be translocated, as TMDs is believed to provide the specificity for the substrate.

NBD, also known as nucleotide-binding fold (NBF) is a site for ATP binding. The NBDs are located in the cytoplasm and have a highly conserved sequence. Therefore, these proteins are classified as ABC transporters based on the sequence and organization of their ATP-binding domain(s).

ABC transporters are either full transporters or half transporters. The structural architecture of full ABC transporters consists minimally of two TMDs and two NBDs, while half transporters consist of one TMD and one NBD domains (Figure 1-2 B). The half transporters must form either homodimers or heterodimers in order to be functional.

ABC transporters are an active transporter, which use the energy of ATP to drive conformational changes in the transmembrane domain (TMD) and consequently

transports molecules (Hollenstein et al. 2007), In eukaryotes, they mostly act as effluxers, moving substrates from inside to outside of the membrane (Dean et al. 2001). These proteins are found to be located in the membranes of healthy cells where they facilitate the transport of various endogenous and exogenous substances.

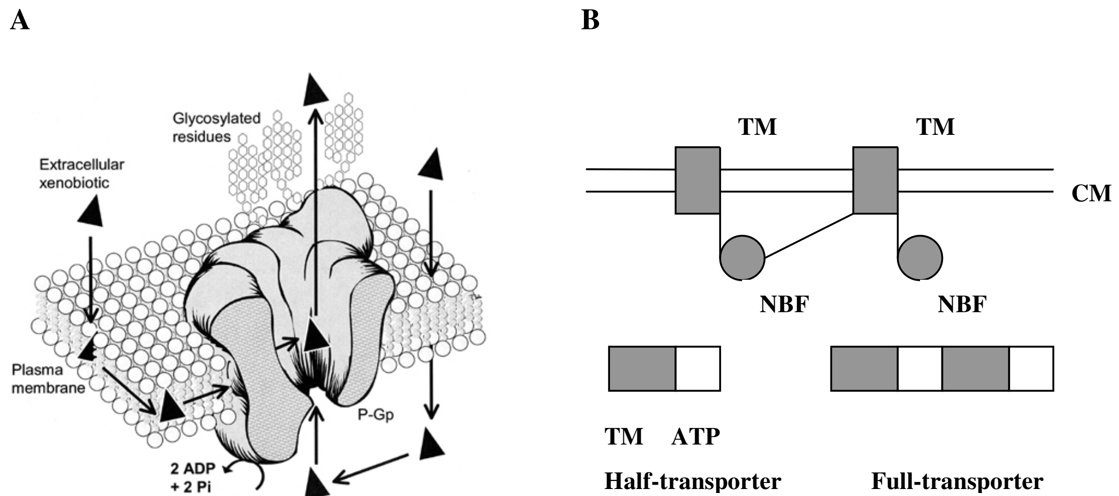

Figure 1-2 **A.** Diagram of the typical structure of an ABC gene (Marzolini et al. 2004) [Reprinted, with permission]. **B.** Structure of half and full transporters (CM: cellular membrane) [figure adopted from Dean et al. (2001)].

For the current study four members of the human ATP-binding cassette (ABC) transporter family, namely ABCB1, ABCC1, ABCC2, and ABCG2 were of particular interest because they are known to be highly expressed in tissues important for absorption (e.g., lung and gut), metabolism and elimination (liver and kidney). For instance, these ABC transporters limit the absorption of many drugs from intestine, and pump drugs from liver cells to bile. Therefore are capable to modulate the absorption, distribution, metabolism, excretion, and toxicity of xenobiotics in intra and extra-cellular environments. These proteins are also active effluxers in barrier site tissues (e.g., blood-brain barrier, blood-cerebral spinal fluid barrier, blood-testis barrier and the maternal-fetal barrier, placenta). Moreover, many drugs are substrates

for these transporters, thus are important in pharmacokinetics of various drugs. ABC drug transporters have their own unique profiles, but ABCB1, ABCC1, and ABCG2 were reported to have overlapping tissue distribution and the substrate specificities (Gottesman, 2002). In pregnancy, the risk of maternal pharmacotherapy on the fetus can be influenced by the performance of these transporters. Collectively, it seems that these ABC transporters can play a crucial role during pregnancy in reducing the level of xenobiotics in maternal and fetal circulation, thereby protecting the developing fetus from exposure to potentially toxic compounds. There is substantial evidence that the four efflux pumps have overlapping functions in tissue defense.

Recently, study of polymorphisms in ABC genes has greatly been regarded. There are numerous sequence variations in genes of the ABC transporter family. However, functional genomic studies and investigation of signatures of recent positive selection (RPS) have contributed to identify a number of functional Single Nucleotide Polymorphisms (SNP) within the genes. Functional SNPs that result in changes in mRNA or protein expression, protein activity, substrate specificity, or influence on bioavailability and therapeutic outcomes can increase susceptibility of individuals to the adverse effects of espousing to xenobiotic and interindividual differences in drug response. Therefore, genetic variation in these genes can be the cause or contributor to a wide variety of human disorders with Mendelian and complex inheritance.

The following discussion and literature reports will be limited to aforementioned ABC transporters and important SNPs of them that are found to alter function or expression of the proteins in organs with absorption, elimination, or barrier function.

Table 1 Function and expression of ABCB1, ABCC1, ABCC2, and ABCG2 genes in human

Name	Alternative titles	Chromosomal Location	Tissue Distribution	Location in plasma membrane	Function	Disorders	Substrates	Inhibitors
ABCB1	MDR1 PGP GP170 Doxorubicin Resistance	7q21	Brain, Kidney, Adrenal, Small Intestine, Testis, Lung, Blood–brain barrier (capillary endothelium), lymphocytes, Placenta (syncytiotrophoblasts)	Apical	Multidrug Resistance		Doxorubicin, daunorubicin, paclitaxel, topotecan, vinblastine, tamoxifen, etoposide, cefazolin, cefoperazone, erythromycin, verapamil, diltiazem, phenytoin, phenoxazine, perphenazine, domperidone, tacrolimus, ritonavir, saquinavir, indinavir, dexamethasone, aldosterone, hydrocortisone, morphine, loperamide, quinidine, digoxin, amiodarone, Cyclosporin A (low concentration),	Reserpine, colchicine, xanthene, phenoxazine, acridine, chloroquin, phenothiazine, ketoconazole, Cyclosporin A (high concentration) Ritonavir (high concentration)
ABCC1	MRP1	16p13	Ubiquitous	Basolateral	Multidrug Resistance LTC4 transport		Vincristine, Vinblastine, Daunorubicin, Doxorubicin, Epirubicin, Etoposide, Imatinib, CPT-11, SB-38, Arsenite, Colchicine, Methotrexate, Mitoxantrone, Saquinivir	MK571, Biricodar
ABCC2	MRP2 cMOAT	10q24	Intestine, Liver, Kidney, Placenta (syncytiotrophoblasts), Blood–brain barrier (capillary endothelium)	Apical	Organic anion transport	Dubin-Johnson syndrome	Vincristine, Vinblastine, Doxorubicin, Epirubicin, Etoposide, Docetaxel, Paclitaxel, CPT-11, SN-38, Topotecan, Cisplatin, Arsenite, Methotrexate, Mitoxantrone, Saquinivir	
ABCG2	BCRP ABCP MXR	4q22	Intestine, liver, Testis, Breast, Placenta (syncytiotrophoblasts), Blood-brain barrier (capillary endothelium)	Apical	Multidrug Resistance		Doxorubicin, Daunorubicin, Epirubicin, Etoposide, Gleevec, Flavopiridol, CPT-11, SN-38, Topotecan, Bisantrene, Methotrexate, Mitoxantrone, AZT	Elacridar, Tariquidar, Biricodar (VX-710)

1.2 ABCB1 (MDR1/ P-glycoprotein)

1.2.1 Function and expression

The human ABCB subfamily (MDR-ABC transporters) consists of 11 members, ABCB1 through ABCB11. Among these, ABCB1 (MDR1) is one of the largest proteins, consisting of about 1280 amino acids. The ABCB1 protein with a molecular weight (MW) of 170-kDa, is also referred to as P-glycoprotein (P-gp) and the gene is designated as *ABCB1*/*MDR1*. Human ABCB1 is found in the epithelia of many tissues, such as intestine, liver, kidney, blood-brain barrier, testis, lung, lymphocytes, hematopoietic stem cells, ovaries, and placenta (Figure 1-3). However, there is variability of expression of this transporter among tissues and within tissues among different population of cells. The expression of ABCB1 is modified by both genetic polymorphism and exogenous factors (drugs, diet) caused a broad interindividual variability.

ABCB1 serves not only a crucial physiological role in protecting the cells against toxic compounds and metabolites, but it also plays a large role in distribution and elimination of many clinically important therapeutic substances. Substrates transported by ABCB1 include metabolic products, lipids, sterols, drugs (such as drugs used in cancer chemotherapy, hypertension, infection, allergy, immunosuppression, neurology, and inflammation) and other xenobiotics. Expression of ABCB1 in the endothelial cells of brain capillaries ,the blood-brain barrier, gives protection against penetration of toxic substances and drugs into the brain. Until now, attentions have been paid to the possible role of *ABCB1* mutations in the pathogenesis of Alzheimer's and Parkinson’s disease.

It can be hypothesized that during pregnancy, ABCB1 in lung, small intestine, kidney, liver, and placenta is able to actively control drugs and other xenobiotics levels in maternal and subsequently fetal circulation, thereby limiting fetal exposure to potentially teratogenic substances. Hence, it is possible that genetic polymorphisms leading to variability in the transporter expression can cause interindividual variability in the fetal exposure at least to some extent. Studies of human term placenta have shown that ABCB1 is expressed at relatively high levels in the brush border (apical) of the syncytiotrophoblast but not in endothelial cells (Atkinson et al. 2003).

It is known that the first trimester of gestational age is highly critical for the risk of embryonic exposure to potential teratogens and developing complex inherited abnormalities mediated by xenobiotics. Interestingly, in human, the mean expression of ABCB1/P-gp in placenta measured by Western blotting method in early (13-14 gestation week) compared to the late (full-term) placentas was found to be two-times higher (Gil et al. 2005). Consistent with this report, Mathias et al. (2005) and Sun et al. (2006) have observed that the human placental P-gp expression appears to be up-regulated in early pregnancy to protect the fetus from xenobiotic toxicity at a time when it is most vulnerable to such toxicity. Mathias et al. (2005) also reported that the decrease in P-gp expression in the term placenta could be related to human chorionic gonadotropin-β (HCG-β) expression.

Additionally, studies in mice offer convincing evidence that P-glycoprotein plays an important role in protecting the fetus from xenobiotics. In an experiment performed by Lankas et al. (1998) in a subpopulation of the CF-1 mouse strain that contains spontaneous mutation in mdr1a gene revealed when pregnant dams were exposed to the teratogenic isomer of avermectin, a known substrate of P-gp, fetuses deficient in

P-gp *ABCB1*a (−/−) gene were 100% susceptible to cleft palate. Whereas, their (+/−) littermates were less sensitive (30% of fetuses with cleft palate) and homozygotes (+/+) fetuses with abundant P-gp were totally insensitive at the tested doses. The authors of the above study confirmed that the absence of the P-glycoprotein in the knock-out mice permitted greater amounts of antihelmintic avermectin, which is known to produce cleft palate in mice, to pass the placental barrier and induce significant cleft palate. They observed that the degree of chemical exposure of fetuses within each litter is inversely related to the expression of placental P-gp. Similarly, Smit et al. (1999) demonstrated that mice in which both the *ABCB1* genes, *ABCB*1a and *ABCB*1b, were disrupted had a 2.4-, 7-, and 16-fold higher transplacental transport of ABCB1 substrates such as digoxin, saquinavir, and paclitaxel, respectively, as compared with wild-type mice.

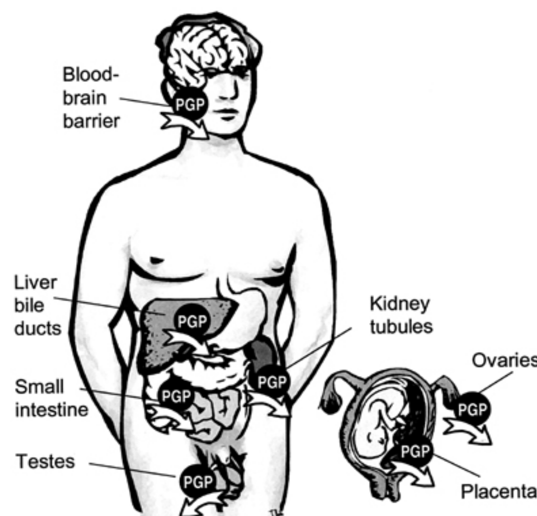

Figure 1-3 P-gp tissue distribution; Efflux activity associated with P-gp would reduce intestinal drug absorption while enhancing drug elimination through the liver and kidney. At barrier sites such as the blood-brain barrier, testes, or placenta, P-gp would limit tissue exposure to potentially toxic P-gp substrate compounds (Marzolini et al. 2004) [Reprinted, with permission].

1.2.2 *ABCB1* gene polymorphisms

The human *ABCB1* gene is located on chromosome 7 and is composed of 28 exons. Although the variable expression of *ABCB1* gene may be caused by different environmental factors, genetic polymorphisms of this gene are believed to represent the major source of the (interindividual) variability in the protein expression.

Thus far, significant progress has been made in the discovery of *ABCB1* polymorphisms and the assessment of allelic frequencies. To date, about 62 single-nucleotide polymorphisms (SNPs) have been identified in the human *ABCB1* gene, including coding and noncoding regions of the gene [http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=5243]. It has been found that important single nucleotide polymorphisms (SNPs) and SNP haplotypes within this gene are variously associated with differences in *ABCB1* expression, drug response, and disease susceptibility. Among discovered SNPs, three variations in exon 21 (G2677T/A), exon 26 (C3435T), and exon 12 (C1236T) are of particular interest because they are in linkage disequilibrium, thereby forming a SNP haplotype which seems to be observed across all ethnic groups (Kim et al. 2001; Tanabe et al. 2001; Horinouchi et al. 2002). These three SNPs occur at high frequency (>10% minor allele frequency) in African-Americans, Caucasians, Chinese, Malays, and Indians. However, differences in the alleles' frequency of these SNPs have been reported in the different ethnicities.

Interethnic differences of these polymorphisms are given in Table 2.

Table 2 Allele frequencies of *MDR1* exon 26, 21, and 12 polymorphisms in various ethnic populations (Marzolini et al. 2004) [Reprinted, with permission].

		3435 (exon 26)		2677 (exon 21)			1236 (exon 12)		
Population	**No.**	**C**	**T**	**G**	**T**	**A**	**C**	**T**	**Study**
Caucasians									
Caucasian, Italy	106	0.54	0.46	0.56	0.41	0.03			Furuno et al
Caucasian, United States	37	0.46	0.54	0.54	0.46	ND	0.58	0.42	Kim et al
Caucasian, Germany	188	0.52	0.48				0.62	0.38	Hoffmeyer et al
Caucasian, Germany	461	0.46	0.54	0.56	0.42	0.02	0.59	0.41	Cascorbi et al
Caucasian, Germany	67	0.49	0.51	0.56	0.40	0.04	0.66	0.34	Siegmund et al
Caucasian, Germany	537	0.50	0.50						Schaeffeler et al
Caucasian, Germany	50	0.49	0.51	0.52	0.38	0.10			Gerloff et al
Caucasian, Poland	103	0.51	0.59						Drozdzik et a
Caucasian, Russia	290	0.46	0.54	0.55	0.42	0.03			Gaikovitch et al
Caucasian, Spain	408	0.52	0.48						Bernal et al
Caucasian, United Kingdom	200	0.47	0.53						Siddiqui et al
Caucasian, United Kingdom	190	0.48	0.52						Ameyraw et al
Caucasian, New Zealand	160	0.47	0.53						Roberts et al
Portuguese	100	0.43	0.57						Ameyaw et al
Asians									
Japanese	50	0.57	0.43						Schaeffeler et al
Japanese	48	0.51	0.49	0.36	0.42	0.22	0.35	0.65	Tanabe et al
Japanese	114	0.61	0.39						Sakaeda et al
Japanese	117	0.62	0.38	0.44	0.36	0.20			Horinouchi et al
Japanese	13	0.54	0.46	0.39	0.46	0.15			Moriya et al
Chinese	132	0.53	0.47						Ameyaw et al
Chinese	96	0.47	0.53	0.38	0.50	0.12	0.28	0.72	Chowbay et al
Chinese	104	0.60	0.40	0.50	0.44	0.06	0.31	0.69	Tang et al
Chinese	98	0.46	0.54						Balram et al
Malay	92	0.49	0.51	0.53	0.44	0.03	0.34	0.66	Chowbay et al
Malay	93	0.63	0.37	0.58	0.36	0.06	0.31	0.69	Tang et al
Malay	99	0.48	0.52						Balram et al
Indian	87	0.37	0.63	0.33	0.60	0.07	0.33	0.67	Chowbay et al
Indian	68	0.40	0.60	0.34	0.62	0.04	0.40	0.60	Tang et al
Indian	93	0.38	0.62						Balram et al
Filipino	60	0.59	0.41						Ameyaw et al
Saudi	96	0.55	0.45						Ameyaw et al
Southwest Asian	89	0.34	0.66						Ameyaw et al
Africans									
Ghanaian	172	0.90	0.10						Schaeffeler et al
Ghanaian	206	0.83	0.17						Ameyaw et al
Kenyan	80	0.83	0.17						Ameyaw et al
Sudanese	51	0.73	0.27						Ameyaw et al
African American	41	0.78	0.22						Schaeffeler et al
African American	88	0.84	0.16						Ameyaw et al
African American	23	0.74	0.26	0.85	0.15	ND	0.85	0.15	Kim et al

***ND, Not done.**

Linkage studies have revealed that in Chinese, Malays, Indians and Caucasians but not African Americans, the T allele of the SNP e26/3435 is frequently linked with the T alleles of the SNPs e21/2677(G/T/A) and e12/1236(T/C). The SNP haplotype C1236T-G2677T-C3435T (i.e., T1236-T2677-T3435) is frequent in European-Americans, whereas the SNP haplotype C1236C-G2677G-C3435C (i.e., C1236-G2677-C3435) is frequent in Africans (Kim et al. 2001; Tang et al. 2002; Ozawa et al. 2004). Functional genomic studies have disclosed that these variations are associated with altered function and expression of P-gp. The two SNPs in exon 26 and 12 are located at a nonpromoter position in the *ABCB1* gene but occur in the functionally important ATP-binding regions (NBD) of the protein (Figure 1-4).

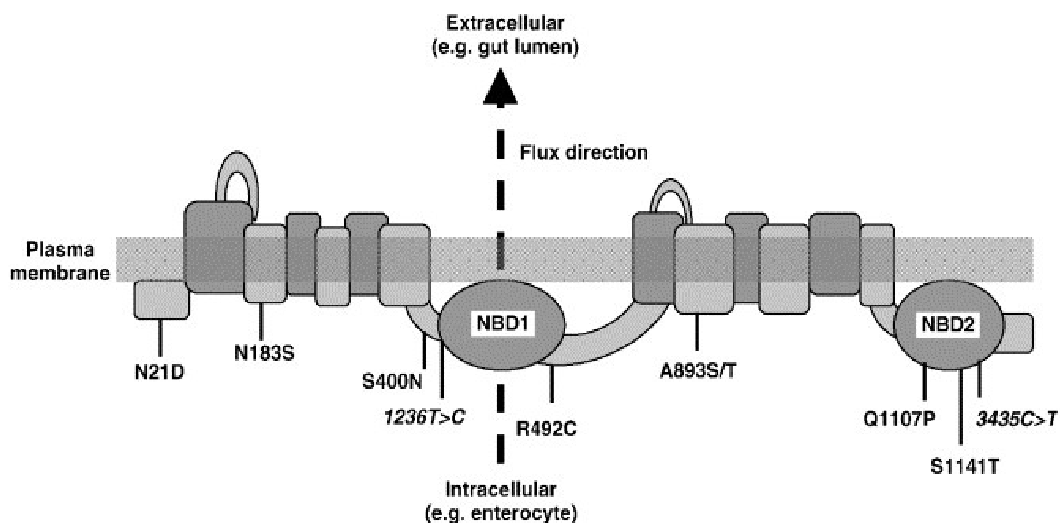

Figure 1-4 Two-dimensional structure of ABCB1 with locations of amino acid replacements and two frequent synonymous SNP (italic); NBD=nucleotide-binding domain (Cascorbi et al. 2001) [Reprinted, with permission].

However, C1236T and C3435T polymorphisms are silent SNPs, as they do not change the amino acid sequences of the encoded protein. The C1236T polymorphism changes a GGC codon to GGT at amino acid position 412 (both encode Gly), and the C3435T

polymorphism changes ATC to ATT at position 1145 (both encode Ile).

The SNP in exon 21 at position 2677 is a nonsynonymous polymorphism that results in two distinct amino acid changes, namely, Ala893Ser (G2677T) and Ala893Thr (G2677A). The C3435T and C1236T SNPs were found to be linked to the nonsynonymous SNP e21/2677(G/T/A), suggesting that functional differences in P-gp attributed to the synonymous SNPs (exon 26 and 12) may be due to the nonsynonymous SNP in exon 21 (G2677T/A) (Tanabe et al. 2001). Moreover, an interesting study by Kimchi-Sarfaty et al. (2007) has showed the way that the synonymous SNP C3435T can still be functional. The author favored a hypothesis that the polymorphism represents a codon, which is rarer than that of the wild-type *ABCB1*, causing a translation pause and an alteration in the rate of translation. Therefore, even in similar mRNA and protein levels observed for the wild type and polymorphic gene, the polymorphism can alter the structure of substrate and inhibitor interaction sites in the protein.

The synonymous SNP in exon 26 (C3435T) was first shown to be associated with decreased expression of P-gp in the duodenum of subjects with the T allele (variant) compared to those with the C allele (wild type) (Hoffmeyer et al. 2000). However, subsequent studies suggested that the decreased expression may be due to the nonsynonymous SNP in exon 21 (G2677T/A) (Kim et al. 2001). Consistent with this finding, Ameyaw et al. (2001) observed marked differences in the allele frequency of the exon 26 SNP (C3435T) between African and Caucasian-Asian populations. The authors reported the allelic frequency of C allele (wild type) is high in African populations (80%) compared to that in Caucasian-Asian populations (45-55%). Because the C allele was initially found to be associated with a higher expression of P-gp, it was hypothesized that the observed higher frequency of the CC genotype in

Africans may have resulted from a selective advantage offered by this genotype against gastrointestinal tract infections (Marzolini et al. 2004).

In another study, Johne et al. (2002) reported increased digoxin plasma concentrations in TT carriers of subjects, suggesting greater intestinal drug absorption due to low intestinal P-gp levels. They also demonstrated the effect was stronger in 2677GG/3435TT carriers. But many later findings were not consistent with this earlier findings, such as reports of elevated expression of ABCB1 mRNA in Japanese subjects carrying the 3435T allele (Nakamura et al. 2002) or Caucasian subjects carrying the 3435T allele (Siegmund et al. 2002). Conflicting data have also been reported for the effect of exon 21 SNP (G2677T/A). However, one explanation for these discrepancies might be haplotype diversities observed for the SNP C3435T with the two other SNPs in exon 12 and 21 among different populations.

In human liver, Wang* et al. (2005) have showed that the mRNA expression of the 3435C allele is significantly higher than that of the 3435T allele. However, they found no association between C1236T and G2677T/A SNPs with lower mRNA levels. The authors have concluded that the C3435T SNP may change mRNA stability and therefore mRNA expression in the liver. In addition, Song et al. (2006) observed that not only the SNP 3435C but also the 2677G allele was associated with higher hepatic ABCB1 mRNA expression and interestingly the GC haplotype pair carriers had a significantly higher hepatic ABCB1 mRNA expression.

In contrast, another study on non-cancerous kidney cortex tissues by Haenisch et al. (2007) revealed that homozygous samples for the SNP 3435C had lower ABCB1 mRNA/18S rRNA ratio compared to heterozygotes or homozygotes 3435T allele carriers. Moreover, they reported that another *ABCB1* polymorphism at

e21/2677(G/T/A) showed a clear correlation with the mRNA expression. The authors observed samples carrying at least one 2677T/A allele had higher ABCB1 mRNA expression levels compared to samples homozygous for 2677G. Likewise, Meissner et al. (2004) reported in human heart tissue the 2677T/A variant was associated with elevated ABCB1 mRNA levels. However, in placenta findings are different. Tanabe et al. (2001) by Western blotting in 100 full-term placentas obtained from Japanese women have found that individuals having the G2677(A,T) allele has less placental trophoblast ABCB1 expression. They also reported that 93.8% of their subjects who had a C3435T allele, also were carrier of a mutant allele from the SNP G2677(A,T), suggesting an association between the two polymorphism. In addition, the authors observed an association between greater placental ABCB1 expression in homozygous CC samples for SNP C3435T compared to CT and TT genotypes, but they attributed this finding to the nonsynonymous SNP G2677(A,T). However, according to a recent study by Kimchi-Sarfaty et al. (2007), the SNP C3435T can be functional regardless of its linkage to the SNP G2677(A,T). Moreover, Hitzl et al. (2004) reported that the placental P-gp expression was lower when both mother and child were carriers of 3435T compared to levels obtained for pairs of 3435C, although they found no influence on the mRNA levels by these polymorphisms. They also found that term placentas from mothers carrying both polymorphisms (3435T and 2677T; TT/TT) had a significantly lower P-gp expression compared to placentas of wild-type individuals.

Nonetheless, as ABCB1 expression is thought to decrease with advancing of gestational age, these two studies would have been more productive if the authors had included preterm placentas in their studies instead of placental tissues from late normal pregnancies. Furthermore, Sherif et al. (2007) have observed that term but not preterm placentas, which are carriers of 2677T/A (homozygous or heterozygous) have

significantly less P-gp expression compared to homozygous placentas for 2677G.

ABCB1 SNP C1236T also found to be functionally important. In Asian subjects, contrary to Caucasians, the T allele of this SNP is more frequent than the C allele. Moreover, the number of observed haplotype combinations is different among diverse populations (Tang et al. 2002). In a study by Mathijssen et al. (2003), upon administration of Irinotecan to 65 cancer patients, the homozygous TT individuals for this polymorphism demonstrated a significant association with increased exposure to irinotecan and its active metabolite SN-38. In addition, Aird et al. (2007) have showed in 3T3 isogenic fibroblasts, mutation at position 1236 can significantly alter *ABCB1* expression, regardless of additional mutations at positions 2677 and 3435. Consistent with these reports, recently Vaclavikovaa et al. (2008) observed that breast cancer patients with variant alleles in exons 12 (1236) and 26 (3435) had significantly lower ABCB1 expression in the tumor.

Table 3 Positions, sequences, and frequencies of *MDR1* variants in human placentas (cDNA) and genomic DNAs and genomic DNAs [adopted from Tanabe et al. (2001)]

SNP	Exon/Position	Nucleotide Sequence		Effect	Placental cDNA (n = 100)					Genome DNA (n = 48)				
		Wild Type (WT)	Mutation (M)		Genotype			Allele Frequency		Genotype			Allele Frequency	
					W/W	W/M	M/M	W	M	W/W	W/M	M/M	W	M
								%					%	
T1236C	12/1236	agggTctga	agggCctga	Gly412Gly	50	31	19	65.5	34.5	21	20	7	64.6	35.4
G2677T	21/2677	aggtGctgg	aggtTctgg	Ala893Ser	14 (G/G)	35 (G/T)	16 (T/T)	43.0	39.0 (T)	9 (G/G)	8 (G/T)	10 (T/T)	36.5	41.7 (T)
G2677A			aggtActgg	893Thr		23 (G/A)	1 (A/A) 11 (A/T)		18.0 (A)		9 (G/A)	0 (A/A) 12 (A/T)		21.8 (A)
C3435T	26/3435	agatCgtga	agatTgtga	Ile1145Ile	35	46	19	58.0	42.0	14	21	13	51.0	49.0

1.3 ABCC1 (Multidrug-resistance related protein 1/MRP1)

1.3.1 Function and expression

The human ABCC subfamily (MRP-ABC transporters) consists of 12 members, ABCC1 through ABCC12. Of these, ABCC1 (MRP1) and ABCC2 (MRP2/cMOAT) are of interest in the current study. MRPs have three hydrophobic transmembrane domains (TMDs) which are also referred to as membrane-spanning domains (MSDs). ABCC1 is a large protein encoded by the *ABCC1* gene, initially identified in the doxorubicin-resistant small cell lung carcinoma cell line H69AR that did not overexpress P-gp (Cole et al. 1992). The 190 kDa -ABCC1 protein is frequently highly overexpressed in multidrug resistant human tumor cell lines, usually as a result of amplification of the *ABCC1/MRP1* gene. This protein confers resistance to a spectrum of anticancer drugs similar to ABCB1. However, unlike ABCB1, ABCC1 transports drugs conjugated to glutathione. Moreover, ABCC1 serves as multispecific organic anion transporter for folate-based antimetabolites, anthracyclines, plant alkaloids and antiandrogens (Conseil et al. 2005).

ABCC1 is expressed in most tissues with relatively high levels found at the blood-brain barrier, blood-testes barrier, placenta, lungs, kidneys, skeletal muscle, spleen, and mononuclear cells and relatively low levels in the liver (Cole et al. 1998). As discussed on ABCB1, ABCC1 is also involved in protecting cells within the body against drugs, environmental toxins, and heavy metals. Moreover, ABCC1 has also been shown to contribute to cellular anti-oxidative defense system and inflammation (Hipfner et al. 1999; Leslie et al. 2001). In most tissues, ABCC1 is localized to the basolateral cell membrane. Thus, it pumps its substrates into the interstitial space

rather than excreting them into the bile, urine, or gut (Borst et al. 1999; Borst et al. 2000). Because of the basolateral expression of ABCC1 this transporter could be important for transport of endogenous substrates, including nutrients (e.g., folic acid) and hormones (e.g., steroid conjugates) (Hooijberg et al. 2004; Ifergan et al. 2004). In the placenta, ABCC1 is predominantly expressed in fetal blood vessel endothelial cells but less on the basolateral surface of the syncytiotrophoblast structure. Being in such strategic locations, ABCC1 is able to prevent or limit entry of organic anions and xenobiotics, such as antiepileptic drugs into the fetal circulation.

Moreover, ABCC1 plays an important role in nutrient absorption and protection of the trophoblast from toxic fetal waste products such as bilirubin and conjugated bile salts (St-Pierre et al. 2000; Atkinson et al. 2003; Nagashige et al. 2003; Gennuso et al. 2004) (Figure 1-1). The analysis of human first and third (term) trimester placenta disclosed that the placental ABCC1 had a four-fold increase in the third as compared with first trimester placental samples (Pascolo et al. 2003).

As discussed previously, folic acid deficiency can increase the risk of orofacial clefting. It can therefore be hypothesized that the impaired role of the placental ABCC1 in nutrient absorption may increase the risk of facial malformations. In addition, placental ABCC1 like ABCB1 is able to give protection to the fetus against antiepileptic drugs and other potentially toxic xenobiotics that increase the risk of orofacial clefts.

1.3.2 *ABCC1* gene polymorphism

The human *ABCC1* gene is located on chromosome 16 and is composed of 31 exons and 30 introns. Thus far, more than 70 SNPs have been identified in exonic, intronic

and promoter regions of the gene in individuals from five different populations, namely, Chinese, Malays, Indians, European Americans, and African Americans. Of these SNPs, a few have showed functional consequences, which might account, in part, for inter-individual and population differences in response to various drugs. Non-synonymous SNPs identified at *ABCC*1 are less than 10%; however, synonymous or intronic SNPs may still affect ABCC1 expression or function through the alteration of the mRNA splicing, stability or folding. Additionally, polymorphisms at the 5'UTR and 3'UTR may influence promoter activity and hence gene expression or mRNA transcript stability.

For the current study, SNP 5'FR/G-260C, which resides in the promoter region of *ABCC1* was selected as target SNP. SNP analysis by Wang et al. (2005) has showed evidence of recent positive selection (RPS) for this polymorphism in European-Americans. In their study on different ethnicities, among approximately 480 individuals sampled from Chinese, Malay, Indian, European-American, and African-American populations, high haplotype frequency extended haplotype homozygosity was observed for allele G of the SNP in European-Americans. However, the authors found weak linkage disequilibrium (LD) in the presence of the high haplotype diversity, suggesting a genomic evidence of positive selection for SNP 5'FR/G-260C in the European-Americans. Furthermore, *in vitro* promoter-reporter assay strikingly revealed that the G-containing promoters had significantly lower promoter activity compared to those carrying the C allele, confirming the functionality of this SNP.

1.4 ABCC2 (Multidrug-resistance related protein 2/ MRP2)

1.4.1 Function and expression

ABCC2 with a MW of 190 kDa and 1545 amino acids is the largest MRP encoded by the *ABCC2* gene. The protein is localized in the apical luminal membrane of polarized epithelial cells of several excretion organs like liver, intestine, and kidney, as well as in blood-brain barrier and placenta (Table 1).

Studies in hepatocytes have shown that ABCC2/MRP2 is involved in exporting a variety of both conjugated and unconjugated anionic compounds into bile. Moreover, the protein was found to be involved in multi drug resistance in diverse tissues. The substrate specificity of ABCC2 is similar to that of ABCC1, and it includes glutathione conjugates such as bilirubin glucuronides, and a number of conjugated drug metabolites. Previous studies have also shown that Dubin-Johnson syndrome (DJS) ,an autosomal-recessive disorder, is linked to the absence of ABCC2 in human liver, resulting in symptomatic conjugated hyperbilirubinaemia. Affected individuals have life-long low-grade jaundice, which may be aggravated by alcohol, pregnancy, infection, and other environmental factors.

In human placenta, ABCC2 is also localized at the apical membrane of syncytiotrophoblasts (facing maternal blood). ABCC2 is likely responsible for preventing the passage of conjugated metabolites of drugs, xenotoxins, and endogenous toxins (e.g., bilirubin) from maternal to fetal circulation. In addition, ABCC2 is potentially important for the passage of conjugated toxic fetal waste products from fetal to maternal circulation for excretion in maternal bile or urine.

Therefore, the multidrug transporter ABCC2 may be also a functional part of the

"placenta barrier". Contrary to ABCB1, ABCC2 is increasingly expressed with advancing pregnancy, suggesting ABCC2 may be more important than ABCB1 in late pregnancy (Schwabedissen et al. 2005; Sun et al. 2006; May et al. 2008). Therefore, the placental ABCC2 can provide protection to mature fetus against maternal pharmacotherapy and waste products produced by the fetus.

1.4.2 *ABCC2* gene polymorphism

Human *ABCC2* gene contains 32 exons and maps to chromosome 10. Similar to other discussed ABC transporters, polymorphisms of *ABCC2* can result in interindividual differences in transport and excretion activity of this protein.

Among *ABCC2* SNPs, 3 polymorphisms at positions -24, 1249, and 3542 SNP were of interest for this study. These SNPs have previously been examined in previous studies for evidence of functional significance or positive selection.

The SNP C-24T resided in the promoter region of *ABCC2* have showed an effect on *ABCC*2 mRNA expression in normal kidney cortex (Haenisch et al. 2007). The authors observed the homozygous samples of -24C allele had higher median ABCC2 mRNA/18S rRNA ratios than heterozygotes and homozygotes -24T carriers. However, no association found between this *ABCC2* SNP and protein expression in the tissue samples.

In contrast, Wang et al. (2007) have reported human liver samples with CT/TT genotype exhibits significantly higher ABCC2 mRNA expression when compared with liver samples with the CC genotype. They also found evidence of recent positive selection (RPS) for the T allele of this SNP in the absence of multiple test correction. Because SNP e1/C-24T resides at the promoter region of *ABCC2*, the authors also

performed a promoter-reporter assay in four different cell-lines. They found that this polymorphism affected *ABCC2* promoter activity and the RPS T-allele of SNP e1/C-24T mediated higher *ABCC2* promoter activity in several cell-lines tested. However, earlier study by Schwabedissen et al. (2005) failed to show the effect of this promoter SNP on *ABCC*2 expression in human placenta.

The exonic SNP e10/G1249A, which involves a conservative amino acid change (V471I), was reported by Schwabedissen et al. (2005) to result in decreased *ABCC*2 mRNA levels in preterm placentas. The authors found that the 1249 AA genotypes were associated with a lower level of ABCC2 mRNA in the placentas of preterm babies. However, no significant difference of mRNA levels was observed in term placentas, suggesting that the mature fetus can take advantage of better protection against xenobiotics or drugs that are ABCC2 substrates. Moreover, it can be hypothesized that the expression of the placental *ABCC2* might be influenced by some factors other than the genetic polymorphism in term pregnancy, such as maternal or placental hormones that tend to increase in late pregnancy. It would thus be interesting to examine the role of the pregnancy hormones in placental *ABCC2* expression. Moreover, Wang et al. (2007) reported SNP e10/G1249A exhibited RPS when type I error reduction was not performed.

Another ABCC2 SNP (G3542T) localized in exon 25 of the gene, has also showed evidence of recent positive selection in the study of Wang et al. (2007), suggesting that this polymorphism can be potentially functional. This SNP change arginine to leucine, but the functional consequence of this polymorphism has yet to be examined experimentally.

1.5 ABCG2 (Breast Cancer Resistance Protein)

1.5.1 Function and expression

ABCG2 (also called BCRP/ABCP/MXR) is the second member of the G-family of ABC transporters. The protein is a half-size transporter containing a single MSD and NBD, that must homodimerize to acquire transport activity (Kage et al. 2002). ABCG2 was initially found to be overexpressed in a subline of human breast carcinoma MCF-7 cells. Later the protein was also found in a variety of stem cells, which can protect them from exogenous and endogenous toxins. Interestingly, up-regulation of ABCG2 expression has been reported in tissues under low-oxygen conditions, suggesting the protection role of this transporter in cells and/or tissues from protoporphyrin accumulation under hypoxic conditions (Krishnamurthy et al. 2006). In addition to such physiological functions, this protein has a crucial role in intestine, liver, placenta, and the blood-brain barrier. In normal human tissues, ABCG2 has been reported to be expressed at the apical surface of enterocytes in gut, liver hepatocytes, placental syncytiotrophoblast, in the capillaries of blood-brain barrier, breast, and to lesser extent in blood-testis barrier. (Ito et al. 2005; Schwabedissen et al. 2006; Maliepaard et al. 2001; Zhou et al. 2001; Eisenblatter et al. 2002). However, there is no substantial evidence for the expression of ABCG2 in the human kidney. Moreover, this transporter is one of the major ABC proteins reported to play an important role in pharmacokinetic and pharmacodynamic of various drugs. ABCG2 has a high capacity for drug transport and wide substrate specificity, including mitoxantrone, prazosin, anthracyclines, camptothecins, and other therapeutic chemicals (Table 1).

Studies in mice have demonstrated that placental ABCG2 can affect the transfer of

drugs across the placental barrier, suggesting a protecting role for these transporters for fetus against environmental toxicants or drugs ingested by the mother during pregnancy. Similarly, in human placenta, ABCG2 has been reported to be expressed at high levels and functionally active, indicating that ABCG2 can play as important role as ABCB1 in providing a protective role for the fetus (Kolwankar et al. 2005; Ceckova et al. 2006).

Furthermore, Schwabedissen et al. (2006) has observed that the ABCG2 mRNA levels in human placenta at preterm (28±1 weeks) are approximately two times greater than that at term (39±2 weeks), and ABCG2 protein expression exhibits the same pattern as that of ABCG2 mRNA. However, other studies used a small sample size failed to show the changes in ABCG2 (protein or mRNA or both) expression in the placenta with advancing gestation.

It would have been better if Schwabedissen et al. (2006) had measured ABCG2 expression in the placentas of earlier than 28 weeks, because a study in mice has demonstrated that placental ABCG2 expression (protein and mRNA) peaks at mid gestation (Wang et al. 2006). If this also applies to the ABCG2 expression in human placenta, it can be concluded that the differential placental expression of ABCB1, ABCC1, ABCC2, and ABCG2 over the course of pregnancy can provide a compensatory mechanism for protection of fetus at different gestational stages. In other words, ABCB1 and ABCG2 are respectively crucial in early and mid gestation, but ABCC1 and ABCC2 seem to be more important in late pregnancy.

1.5.2 *ABCG2* gene polymorphism

Human *ABCG2* gene contains 15 exons and 16 introns maps to chromosome 4. Widely occurring single nucleotide polymorphisms in ABCG2 may affect on its

physiological function or absorption and distribution of drugs that are ABCG2 substrates. *ABCG2* SNP C421A at exon 5 has been extensively examined to search for its potential functions. This SNP occurs in the functionally important ATP-binding region of the ABCG2 between the Walker A and B motifs, resulting in substitution of the positively charged Lys residue for a neutral Gln residue. Imai et al. (2002) has showed that the C421A polymorphism is associated with significantly lower expression and activity of the encoded protein. However, the authors found no influence on ABCG2 mRNA expression. Moreover, studies have revealed that the SNP C421A can result in changes in the protein structure and alter ATPase activity and drug efflux properties of ABCG2 (Imai et al. 2002; Mizuarai et al. 2004; Morisaki et al. 2005; Kobayashi et al. 2005; Kondo et al. 2004). This SNP has also computationally exhibited an evidence of recent positive selection in the study of Wang et al. 2007.

In Japanese placentas, Kobayashi et al. (2005) observed the mean ABCG2 protein level of the A421 homozygotes was approximately 50% of that of the C421 allele, and heterozygotes displayed an intermediate level. They further showed that this difference was likely caused by post-transcriptional regulation rather than changes in mRNA expression.

Collectively, these findings suggest that the C421A polymorphism can decrease *ABCG2* expression, leading to increased fetal xenobiotics or drug exposure. As shown in Table 4, the alleles' frequency of this SNP greatly differs between diverse populations.

In addition to the above SNP, G34A polymorphism in *ABCG2* notably occurred at a relatively high frequency in most ethnic populations has been implicated to have

potential functional importance. The non-synonymous SNP e2/G34A (Val12Met) was reported to be associated with substrate recognition and/or transport of drugs, suggesting that this polymorphism impairs the specific apical membrane localization of ABCG2 (Tamura et al. 2006; Tamura et al. 2007). Moreover, Mizuarai et al. (2004) have reported that the G34A variant exhibits reduced drug resistance in polarized porcine kidney epithelial LLC-PK1 cells along with increased intracellular drug accumulation.

At last, another target SNP within *ABCG2* for this study was T-357C that occurs in the intron 9 of the gene. Because this SNP is found to change an intronic splice regulatory element (ISRE) and mutations in ISREs can alter the pre-mRNA splicing (Yeo et al. 2007), it can be potentially functional.

Table 4 Frequency of the C421A *BCRP* allele among different ethnic populations (Yanase et al. 2006) [Reprinted, with permission]

Population	N	Genotype		Allele frequency (%)	Reference
		C/A	A/A		
Asian (Japanese)	124	48	9	27	Imai et al. 2002
Asian (Japanese)	120	45	14	30	Kobayashi et al. 2005
(Han Chinese)	95	43	11	34	de Jong et al. 2004
Caucasian	150	25	4	11	Kobayashi et al. 2005
Caucasian	150	22	2	9	Mizuarai et al. 2004
(American)	88	19	1	12	de Jong et al. 2004
(European)	84	14	2	11	de Jong et al. 2004
(Swedish)	60	10	1	10	Backstrom et al. 2003
African (sub-Saharan)	938	14	1	1	de Jong et al. 2004
African-American	94	8	1	5	de Jong et al. 2004
African-American	150	5	1	2	Kobayashi et al. 2005

Abbreviations: *N*, number of patients studied; C/A, heterozygous frequency; A/A, homozygous variant frequency; Ref., reference.

*Data are given as the relative frequency of variant alleles.

Table 5 Summary of selected target SNPs within four ABC Transporter Genes found to be potentially functional (pages 30-32)

Gene	SNP ID	dbSNP (rs)	Amino Acid Change	Reports on Functionality	Reference
ABCB1	**e26/C3435T**	rs1045642	Ile1145Ile	Associate with expression and activity of ABCB1 in duodenum	Hoffmeyer et al. 2000
				Alteration in the rate of translation	Kimchi-Sarfaty et al. 2007
				Associate with increased digoxin plasma concentrations	Johne et al. 2002
				Associate with ABCB1 mRNA expression levels in Japanese subjects (TT>CC)	Nakamura et al. 2002
				Associate with ABCB1 mRNA expression levels in Caucasian subjects	Siegmund et al. 2002
				Associate with ABCB1 mRNA expression levels in liver	Wang* et al. 2005
				Associate with ABCB1 mRNA expression levels in liver	Song et al. 2006
				Associate with ABCB1 mRNA expression levels in kidney	Haenisch et al. 2007
				Associate with ABCB1 expression levels in term placenta	Tanabe et al. 2001
				Associate with ABCB1 expression levels in term placenta	Hitzl et al. 2004
	e21/G2677T/A	rs2032582	Ala893Ser/Thr	Associate with ABCB1 mRNA expression levels in liver	Song et al. 2006

Gene	SNP ID	dbSNP (rs)	Amino Acid Change	Reports on Functionality	Reference
ABCB1	**e21/G2677T/A**	rs2032582	Ala893Ser/Thr	Associate with ABCB1 mRNA expression levels in kidney	Haenisch et al. 2007
				Associate with ABCB1 mRNA expression levels in heart	Meissner et al. 2004
				Associate with ABCB1 expression levels in term placenta	Tanabe et al. 2001
				Associate with ABCB1 expression levels in term placenta	Hitzl et al. 2004
				Associate with ABCB1 expression levels in term placenta	Sherif et al. 2007
	e12/C1236T	rs1128503	Gly412Gly	Associate with increased exposure to irinotecan in cancer patients	Mathijssen et al. 2003
				Associate with ABCB1 expression in isogenic fibroblasts	Aird et al. 2007
				Associate with ABCB1 expression levels in breast tumors	Vaclavikovaa et al. 2008
ABCC1	**5'FR/G-260C**	rs504348	--	Affect *ABCC1* promoter activity	Wang et al. 2005
ABCC2	**e1/C-24T**	rs717620	--	A risk factor for the development of spina bifida	Jensen et al. 2004
				T allele associates with lower mRNA levels in normal kidney cortex	Haenisch et al. 2007
				Affect *ABCC*2 promoter activity	Wang et al. 2007

Gene	SNP ID	dbSNP (rs)	Amino Acid Change	Reports on Functionality	Reference
ABCC2	**e10/G1249A**	rs2273697	V471I	Associate with ABCC2 mRNA expression levels IN preterm placentas	Schwabedissen et al. 2005
	e25/G3542T	rs8187692	R1181L	Has evidence of recent positive selection	Wang et al. 2007
ABCG2	**e5/C421A**	rs2231142	Q141K	Associate with ABCG2 protein expression and protein structural changes	Imai et al. 2002 Mizuarai, et al. 2004 Morisaki et al. 2005 Kobayashi et al. 2005 Kondo et al. 2004
				Has evidence of recent positive selection	Wang et al. 2007
				Associate with ABCG2 expression levels in term placenta	Kobayashi et al. 2005
	e2/G34A	rs2231137	Val12Met	Impairs the specific apical membrane localization of ABCG2	Tamura et al. 2006 Tamura et al. 2007
				Associate with drug resistance in polarized porcine kidney epithelial LLC-PK1 cells	Mizuarai et al. (2004)
	I9/T-357C	rs2054576	--	Change an intronic splice regulatory element	Yeo et al. (2007)

1.6 Objectives of this study

As discussed, *ABCB1*, *ABCC1*, *ABCC2*, and *ABCG2* are highly expressed in tissues important for absorption (e.g., lung and gut) and metabolism (liver) and elimination (liver and kidney). In addition, these transporters have an important role in barrier site tissues (e.g., placenta). Thus, these ABC transporters are capable to modulate the absorption, distribution, metabolism, excretion, and toxicity of xenobiotics in maternal circulation. Moreover, these transporters are found to be highly expressed in placenta and are involved in both materno-fetal and feto-maternal trafficking of exogenous or endogenous substances. Therefore, they are actively involved in absorption of nutrition by the fetus and modulating the level of toxicants, drugs, and their metabolites ingested by the mother in the fetal circulation. It is known that the functional sequence variations in these genes might alter the expression or function of the transporter proteins.

The current study aimed to investigate the role of important SNPs within the four members of ABC-transporter family in non-syndromic orofacial clefting in which disease risk is mediated by environmental toxicants or nutritional insufficiency during embryonic life.

Considering common environmental risk factors for different types of orofacial clefts, for the current study the phenotype of interest included all forms of non-syndromic orofacial clefts including cleft lip (unilateral or bilateral) with or without cleft palate (CL/P), cleft palate (CP), or both. However, the aim of this study was not to investigate the role of different environmental factors in the disease risk.

1.7 Significance of this study

It is important to acknowledge that in human placenta, the understanding of the activity of different ABC transporters is very preliminary. However, the understanding of the association of functional sequence variations in ABC genes known to be expressed in placenta with birth defects and the interactions of environmental factors such as drugs with placental transporters is very important. Because it can provide a novel framework for further studies on drug development and the choice of therapy in pregnancy in which medications can be designed to control the degree of fetal exposure and thus prevent fetal risk. Moreover, it would be helpful to screen pregnancies that are in higher risk because of less placental protection on the fetus to bring them under close observation especially in case of maternal pharmacotherapy or smoking.

1.8 Study design and Genotyping method

This study designed in two formats of family-based and case-control study. Subjects were genotyped for the target genes and SNPs using genomic DNA extracted from peripheral blood. Genotyping was accomplished by multiplex PCR amplification, multiplex minisequencing and TaqMan® SNP genotyping assay. In addition, DNA sequencing as a reliable genotyping method was employed to validate unclear genotyping data.

2 MATERIAL AND METHODS

2.1 Protocol of subject recruitment and Sample size

To estimate the number of case-parent trios needed to detect genes exerting modest (doubling, r=2) to strong (quadrupling, r=4) levels of relative risks, the Quanto program of Guaderman (2002) was used. Table 5 shows the number of trios needed to attain 80% power under different risk models, assuming risk allele frequencies (p_A) of 0.01, 0.1, 0.25, and 0.5. A sample size of ~150 trios will be adequate to detect common genes with moderate (r=2) or large effect (e.g. r=4) when the risk allele frequency (p_A) is 0.1 or higher, assuming a dominant or codominant (i.e. log-additive) genetic model. However, this sample size will be inadequate for risk alleles with a recessive genotypic effect, for which larger sample sizes will be required.

Table 6 Number of parent-child trios needed to achieve 80% power in the TDT with single affected offspring families.

p_A	0.01		0.1		0.25		0.5	
Genetic model	r=2	r=4	r=2	r=4	r=2	r=4	r=2	r=4
Log-Additive ($r_1^2=r_1=r$)	1178	212	141	29	77	19	69	20
Dominant ($r_2=r_1=r$)	1205	221	175	41	127	36	189	63
Recessive ($r_2=r$, $r_1=1$)	146146	24317	1587	271	309	58	129	31

p_A: frequency of risk allele A at SNP locus
r: genotypic relative risk; r_1: relative risk of genotype Aa; r_2: relative risk of genotype AA

Thus, for the study a population of 150 nuclear families with single oral cleft child was recruited through treatment centers in Singapore and Taiwan with prior approval from the Institutional Review Boards (IRB) in both countries. In the families, the parents were all unaffected with oral clefts and the ethnicity of the population study consisted of Chinese (128 families), Malays (10 families), Indians (4 families) and

other ethnicities (8 families). However, the family based studies are robust against differences exist in ethnicities and genetic background.

For the case-control study, two groups of cases separately were compared against a group of unrelated controls. The population of controls consisted of cord blood DNA samples obtained from 189 healthy Chinese mothers. Case group once consisted of 129 Chinese mothers of oral cleft children, and another time cases were 128 Chinese oral cleft children recruited for the family based study. The ethnicities were all determined through case files and hospital records.

2.2 Laboratory Methods

2.2.1 PCR and Minisequencing

A single triplex PCR was designed to amplify the fragments of *ABCB1*, *ABCC1*, and *ABCG2* containing the three SNPs 2677G>T/A, 5'FR/G-260C, and 34G>A, respectively. Moreover, multiplex minisequencing was optimized for genotyping the PCR products at the interested loci. The Operon online software was applied to design primers for both PCR and minisequencing experiments. Information on the designed primers as well as the reaction conditions have been tabulated in Table 7.

Briefly, for the triplex PCR, a 10 µL reaction mixture was prepared containing PCR buffer (Qiagen), 1.5 mM MgCl2, 200 µM each of the four deoxynucleotide triphosphates (dNTPs), 1 U of HotStarTaq® Polymerase (Qiagen), and 0.5 ng of template DNA. The final primer concentrations (Table 7A) were 0.2 µM (for *ABCB1*/exon 21), 0.4 µM (for *ABCG2*/exon 2), and 0.5 µM (for *ABCC1*/5'FR). The reaction mixture was subjected to initial denaturation at 95 °C for 15 min followed by

35 step-cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min. This was followed by a final extension at 72 °C for 5 min. All PCR amplifications carried out in 96-well plates using T1 thermal cycler (Biometra). The expected PCR fragments and their sizes are shown in Table 7A. After PCR amplification, unincorporated dNTPs and excess primers were inactivated and degraded in a single-step reaction by addition of 5 U of exonuclease I and 0.5 U of shrimp alkaline phosphatase (SAP; United States Biochemical), respectively, to 1.3 µL of the PCR product in a final volume of 2 µL. Then, the reaction mixture was incubated at 37 °C for 1h, and the enzymes were subsequently inactivated at 80 °C for 15 min. The treated PCR products were then subjected to a multiplex minisequencing reaction to interrogate the three SNP loci simultaneously.

Minisequencing primers were designed to anneal to template DNA next to each SNP site such that extension by DNA polymerase added a single dideoxyribonucleoside triphosphate (ddNTP) complementary to the nucleotide at the polymorphic site. Table 7B details the sequences of the minisequencing primers and their concentrations in the final minisequencing reaction mixture. The multiplex minisequencing reaction contained the treated triplex PCR product (1.5 µL), various concentrations of minisequencing primers, and 1 µL of SNaPshotTM Multiplex Ready Reaction Mix (Applied Biosystems) in a total reaction volume of 4 µL. The reaction mixture was then subjected to 25 single-base extension cycles of denaturation at 96 °C for 10 s, primer annealing at 50 °C for 5 s, and primer extension at 60°C for 30 s. Thereafter, unincorporated fluorescent ddNTPs were inactivated enzymatically with 0.5 U of SAP at 37 °C for 30 min, followed by SAP deactivation at 80 °C for 15 min. The multiplex minisequencing products (0.8 µL) were then mixed with 9 µL of HiDiTM formamide and 0.1 µL of GeneScan-120 LIZ size standard (Applied Biosystems), and resolved by

automated capillary electrophoresis for 25 min on an ABI PRISM 3100® AVANT Genetic Analyzer (Applied Biosystems, Foster City, CA).

Minisequencing analyses were performed with the GeneScan (V3.7) application software (Applied Biosystems). Samples that did not give a reliable result in the first round of genotyping were resubmitted to up to two additional rounds of genotyping and DNA sequencing was performed whenever the minisequencing was unsuccessful.

Table 7 Multiplex PCR and minisequencing of the ABCB1, ABCC1, and ABCG2 genes.

A. Primers for the triplex PCR of the 3 genomic regions of the ABCB1, ABCC1, and ABCG2 genes

Gene	Amplified Region	5'-Primer	3'-Primer	Conc. (µM)	Amplicon Length (bp)
ABCB1	Exon 21	5'-ATAGCAAATCTTGGGACAGG-3'	5'-GCATAGTAAGCAGTAGGGAG-3'	0.2	256
ABCC1	Promoter	5'-CAGGATGAAATGAGGGCACAG-3'	5'-GAAGCGCCTGGGATCTTTGG-3'	0.5	155
ABCG2	Exon 2	5'-GTTCATAGCCAGTTTCTTGG-3'	5'-GTTCTTATCACAATGGTATGG-3'	0.4	466

B. Primers for the triplex minisequencing of the 3 SNPs within ABCB1, ABCC1, and ABCG2 genes

* Gene * SNP ID * dbSNP (rs)	Location	Region	Nucleotide Sequence		Primer Orientation	Primer Sequence *Italicized letters represent nonhomologous tail at 5' end of SNP primer*	Length (bp)	Conc. (µM)
			Allele 1	Allele 2/3				
ABCB1 2677G>T/A **rs2032582**	Exon 21	TM- 9.10	gaaggt**G**ctggga	gaaggt**T**ctggga gaaggt**A**ctggga	Reverse	*5'-T(GACT)2*TAGTTTGACTCACCTTCCCAG-3'	30	0.26
ABCC1 5'FR/-260G>C **rs504348**	5'FR	Promoter	aaacag**G**atttga	aaacag**C**atttga	Reverse	5'-CCTGCGACCACTTTTCAAAT-3'	20	0.52
ABCG2 34G>A **rs2231137**	Exon 2	-	gtgaca**G**tgggat	gtgaca**A**tgggat	Reverse	*5'-(GACT)*GGTAATGTCGAAGTTTTATCCCA-3'	28	0.13

2.2.2 TaqMan® SNP Genotyping Assays (Applied Biosystems)

The TaqMan assay (Applied Biosystems) was accomplished for SNP analysis in *ABCB1*, *ABCC2*, and *ABCG2* using original DNA samples. Table 8 displays the TaqMan Assay ID for the selected target SNPs within theses genes.

Table 8 Target SNPs for TaqMan Assay

Gene	SNP ID	dbSNP (rs)	TaqMan Assay SNP ID
ABCB1	e12/C1236T	rs1128503	C-7586662-10
	e26/C3435T	rs1045642	C-7586657-1
ABCC2	e1/G-24A	rs717620	C-2814642-10
	e10/G1249A	rs2273697	C-22272980-20
	e25/G3542T	rs8187692	C-25591748-30
ABCG2	e5/C421A	rs2231142	C-15854163-70
	I9/-357T>C	rs2054576	C-11945745-10

The assay was carried out in a volume of 5 µL containing 1X TaqMan Universal PCR Master Mix, 1X SNP Genotyping Assay Mix (primers and fluorescent probes), and 1ng genomic DNA. The reaction mixture was subjected to initial AmpliTaq Gold Enzyme Activation at 95 °C for 10 min followed by 40 step-cycles of denaturation at 92 °C for 15 s, and thannealing at 60 °C for 1 min using T1 thermal cycler (Biometra) for 96-well plates.

Plate read after PCR amplification was performed on the Applied Biosystems 7500 Real-Time PCR System and SDS v1.4 software was used to determine allelic discrimination during a postamplification plate read based on Rn values (the ratio of fluorescence) of the signals from each well.

2.2.3 PCR and DNA Sequencing

Whenever the results of the SNP genotyping by minisequencing or TaqMan assays were not clear enough, genotyping was repeated by sequencing the PCR product of the DNA fragment containing the respective SNP in the forward or reverse direction using the same PCR primers.

Unless for a few samples, the TaqMan SNP genotyping assay for *ABCC2* SNP G1249A was unsuccessful to clearly determine the SNP genotype. Hence, DNA sequencing was performed for samples that the TaqMan assay results was unreliable as follows:

First, the PCR reaction was performed in a 10 µL volume reaction containing 1 ng genomic DNA template, 5 µL 2X PCR master mix buffer (Qiagen, Valencia, CA, USA), 0.5 µM forward primer (5'-GAGCACATCCTTCCATTGTAATC-3'), and 0.5 µM reverse primer (5'-ACTGAGGGTCCCAACTCTCTC-3'). The thermal cycling conditions for PCR using a T3 thermal cycler (Biometra) was as follows: Initial activation at 95 ^{o}C for 15 min followed by 35 cycles of denaturation at 95 for 30 s, primer annealing at 62 °C for 30 s, and primer extension at 72 °C for 30 s. This was then followed by a final elongation step at 72 ^{o}C for 5 min.

Thereafter, the obtained amplification products (281 bp) were treated with exonuclease I and shrimp alkaline phosphatase (SAP, United States Biochemical). Then a 10 µL sequencing reaction mixture using ABI PRISM Big Dye (V3.1) kit was

prepared containing 2.5 µL 1X Sequencing Buffer, 0.5 µL BigDye (V3.1), 0.16 µM of the reveres primer used in the PCR reaction, and the treated PCR product.

Thermal cycling conditions for the above reaction was 94 ^{o}C for 15 min followed by 30 cycles at 96 ^{o}C for 10 sec, 50 ^{o}C for 5 sec and 60 ^{o}C for 4 min. Thereafter, DNA precipitation was performed with EDTA and Ethanol and the final product was resolved by automated capillary electrophoresis on an ABI PRISM 3100® AVANT at 94 for 30 s, primer annealing at 60 °C for 30 s, and primer extension at 72 °C for 30 s. This was then followed by a final elongation step at 72 ^{o}C for 5 min. Genetic Analyzer (Applied Biosystems, Foster City, CA). The GeneScan (V3.7) application software (Applied Biosystems) was applied to read and analyses the data.

To validate the TaqMan assay results for another *ABCC2* SNP in exon 25 (G3542T), PCR amplification and sequencing was carried out as follows:

The PCR reaction was prepared in a 10 µL volume reaction containing 1 ng genomic DNA template, 5 µL 2X PCR master mix buffer (Qiagen, Valencia, CA, USA), 0.4 µM forward primer (5'-ACTCTGTCACCAGGTCCC-3'), and 0.4 µM reverse primer (5'-CCTCCCACCGCTAATATC-3'). The reaction mixture was subjected to initial activation at 95 ^{o}C for 15 min followed by 35 cycles of denaturation. Thereafter, sequencing of the PCR product was performed using ABI PRISM BigDye (V3.1) kit and 0.16 µM of the PCR forward primer. All PCR amplifications and sequencing reactions were carried out using T3 thermal cycler (Biometra).

In addition, PCR and sequencing were used to verify the minisequencing results for the SNPs 2677G>T/A of *ABCB1* and G34A of *ABCG2* whenever the TaqMan genotyping results were unclear. In order for the SNP 2677G>T/A, ABI PRISM BigDye (V3.1) sequencing kit and 0.16 µM of the reverse PCR primer and for the

SNP G34A, the same sequencing kit but 0.16 µM of the forward PCR primer were used.

2.3 Statistical analysis methods

2.3.1 Family-based study

The genotype data of the one hundred and fifty complete trios of non-syndromic oral cleft patients and their biological parents were assessed for the Mendelian consistency within each family as well as for deviations from Hardy-Weinberg equilibrium (HWE) to ascertain the data are reliable and error free. An online calculator [http://www.husdyr.kvl.dk/htm/kc/popgen/genetik/applets/kitest.htm] was used for the HWE analysis. Thereafter, the transmission disequilibrium test (TDT) on the diallelic SNPs and extended transmission disequilibrium test (ETDT) on the tri-allelic SNP 2677 of *ABCB1* were performed using ETDT software developed by Sham and Curtis (1995). The odds ratios as well as 95% confidence intervals were also computed with an online calculator [http://www.graphpad.com/quickcalcs/McNemar1.cfm].

2.3.2 Case-control study

The online calculator [http://www.husdyr.kvl.dk/htm/kc/popgen/genetik/applets/kitest.htm] was applied to evaluate the significance of deviation of each SNP in the study populations from Hardy-Weinberg Equilibrium (HWE). Thereafter, pair-wise comparisons of alleles frequency between two different groups of cases and controls were performed using Fisher's exact test and Chi-squared (df = 2) for the diallelic SNPs and the *ABCB1* tri-allelic SNP 2677, respectively. Moreover, an online program [http://faculty.vassar.edu/lowry/odds2x2.html] was employed to compute the odds ratios and corresponding 95% confidence intervals for each examined SNP.

2.3.3 Integrating TDT and Case-control studies

To obtain a combine estimate for the disease-SNP association from both family based and case-control studies, a joint analysis was performed with the analytical method introduced by Kazeem and Farrall (2005).

3 RESULTS AND DISCUSSION

3.1 Laboratory Results

The results of multiplex PCR and minisequencing optimized for simultaneous genotyping of three SNPs G-260C, G34A, and G2677T/A is illustrated in Figure 3-1.

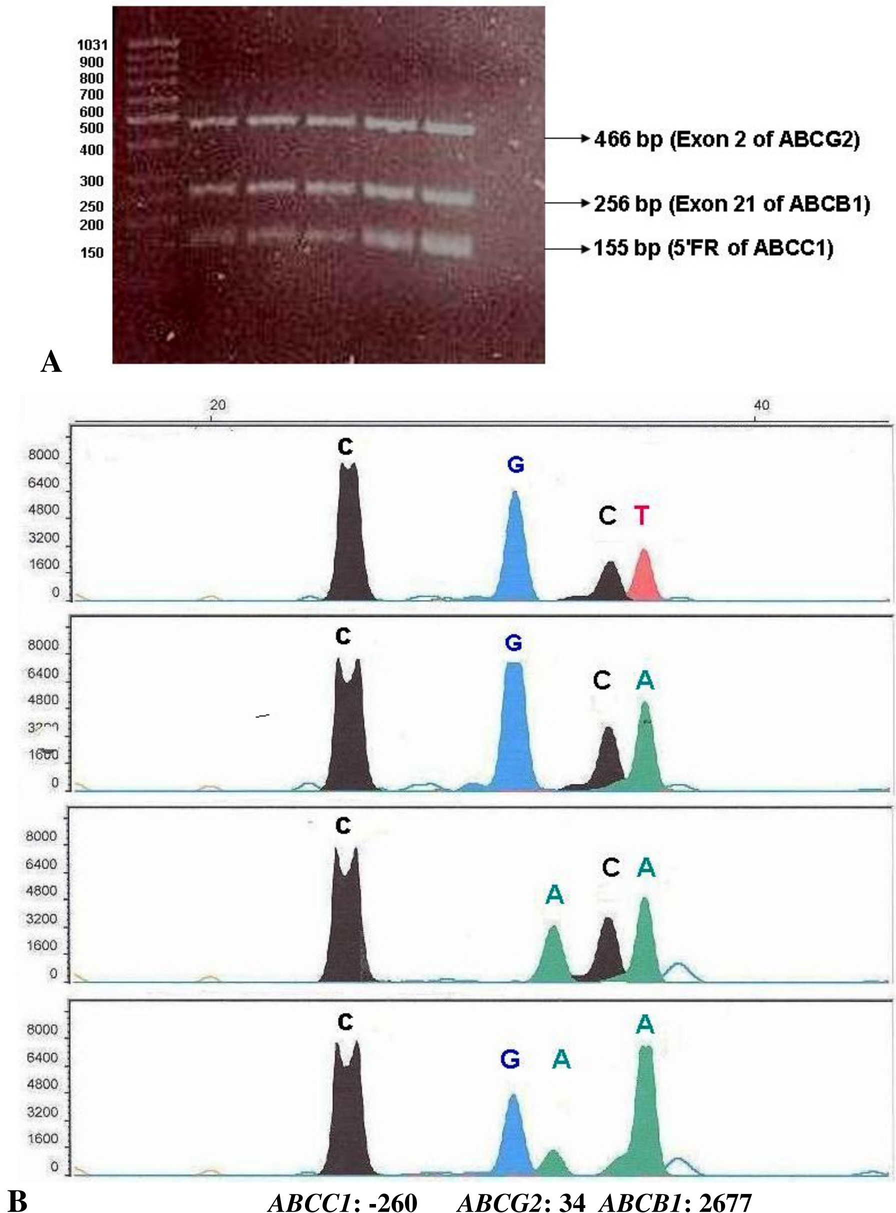

Figure 3-1 Multiplex PCR and genotyping results for the three ABC genes SNPs.
(A), Agarose gel electrophoresis of the multiplex PCR products from five representative samples. A 2-μL aliquot of each reaction product was resolved on a 1.5% agarose gel. **(B),** GeneScan 3.7 analysis of multiplex-minisequencing products. Electropherograms of representative samples with different genotypes at the three SNP sites are shown. Each SNP allele displays a characteristic peak color, position, and height relative to the other allele peak.

The genotyping results of the *ABCC2* SNP in exon 25 (G3542T) for all samples displayed a pattern of monomorphism, therefore this SNP was excluded from further evaluation and the study was limited to the analysis of the rest 9 SNPs in the families, cases and controls .

3.1.1 Results of TDT-McNemar analysis on nuclear families

In parents and probands, the genotyping distributions were all in Hardy-Weinberg equilibrium and no statistically significant deviation was found ($P>0.05$) [Table 11].

Strikingly, the TDT-McNemar analysis revealed that the SNPs within the *ABCB1* gene (e12/1236, e21/2677, and e26/3435), but not SNPs in other ABC genes were significantly associated with orofacial clefting ($P<0.05$). However, after Bonferroni correction, only SNP e12/1236 (rs1128503) remained significantly associated ($P = 0.000051$) with the oral clefts in the family samples while the two SNPs e21/2677, and e26/3435 showed a nominal association (Table 12).

Further allelic-wise analysis using modified McNemar's test showed a preferential transmission of the variant alleles (T) compared to the wild type alleles (C) in the SNPs 12/1236 (C>T) and e26/3435 (C>T). Similarly, as to the tri-allelic *ABCB1* SNP e21/2677(G>T,A), a chi-square statistic (df =1) could show a preferential transmission for the T and A alleles compared to the G allele from heterozygote parents to the probands. However, after correction for multiple testing only the P-value for the T allele remained statistically significant ($P<0.05$) but not for G and A alleles (Table 9).

Table 9 Transmission for individual alleles of SNP e21/2677 (G>T, A)

ABCB1 SNP	e21/2677		
Allele	**A**	**G**	**T**
Number of times passed	23	78	96
Expected number of times passed*	32.5	84.5	80
Number of times not passed	42	91	64
Expected number of times not passed*	32.5	84.5	80
Chi-squared	5.554	1.000	6.400
P-value of chi-squared test (df=1)	0.0185	0.3174	0.0114
Corrected P-value for multiple testing	0.0555	0.9522	0.0342

*Because the expected number of times for each allele to be passed and not passed to the probands is half the total number of times a heterozygote parent has the allele, the expected values calculated accordingly.

A novel analysis method was designed to evaluate the role of the maternal genotype for the target SNPs in vulnerability to orofacial clefting in addition to the fetal genotype. For this analysis, only the oral clefts' families that both parents were Chinese were employed to eliminate the effect of ethnicity and genetic background on the genotype distributions. Then, the proportions of homozygote parents (whether father or mother) for an SNP allele whose oral cleft child was also homozygote for that allele was computed from the genotype data (Table 10). It is plausible that in a normal situation a homozygote child for a specific SNP allele has an equal chance for having a homozygote father or a homozygote mother for that SNP allele. Thus, if the frequency of families with a homozygote oral cleft child for a particular variant SNP allele whose mother is also homozygote for the same allele is significantly greater

than the frequency of families with a homozygote father and child for that SNP allele, it can favor the synergistic effect of the maternal genotype in the disease risk.

As shown in Table 10, a Two Dependent Proportions Testing using an online calculator [http://home.ubalt.edu/ntsbarsh/Business-stat/otherapplets/PairedProp.htm] revealed no significant difference between the frequencies of homozygote father/child genotypes and mother/child genotypes (P>0.05) for all the examined SNPs alleles

Table 10 Proportions of genotype distributions in 129 Chinese families of oral cleft patients when both mother and her child or both father and his child were homozygote for wild type and mutant SNP alleles

Polymorphism	Child/Parent Genotype	Homozygote child and mother proportions	Homozygote child and father proportions	P-value of Two dependent proportions testing
rs1128503 (e12/C1236T)	TT/TT CC/CC	0.290 0.039	0.290 0.031	**-** **0.366**
rs2032582 (e21/G2677T/A)	TT/TT AA/AA GG/GG	0.047 0.000 0.085	0.054 0.000 0.093	**0.402** **-** **0.415**
rs1045642 (e26/C3435T)	CC/CC TT/TT	0.302 0.047	0.225 0.054	**0.114** **0.402**
rs504348 (5'FR/G-260C)	GG/GG CC/CC	0.969 0.000	0.977 0.000	**0.474** -
rs717620 (e1/C-24T)	CC/CC TT/TT	0.504 0.007	0.473 0.000	**0.361** **0.171**
rs2273697 (e10/G1249A)	GG/GG AA/AA	0.760 0.000	0.705 0.000	**0.303** **-**
rs2231142 (e5/C421A)	CC/CC AA/AA	0.333 0.023	0.326 0.031	**0.461** **0.348**
rs2231137 (e2/G34A)	GG/GG AA/AA	0.287 0.023	0.310 0.047	**0.368** **0.152**
rs2054576 (I9/T-357C)	TT/TT CC/CC	0.419 0.016	0.395 0.023	**0.382** **0.344**

Table 11 Genotype and allele frequencies of the polymorphisms in oral cleft patients and biological parents

Polymorphism	No	Genotype						HWE (p)	Allele frequencies		
rs1128503		**TT**	**TC**	**CC**					**T**	**C**	
Parents	300	124	137	39				0.90	0.642	0.358	
Probands	150	79	58	13				0.62	0.720	0.280	
rs2032582		**AA**	**AG**	**AT**	**GG**	**GT**	**TT**		**A**	**G**	**T**
Parents	300	3	37	28	62	132	38	0.13	0.118	0.488	0.393
Probands	150	0	16	10	32	60	32	0.42	0.087	0.467	0.447
rs1045642		**CC**	**CT**	**TT**					**C**	**T**	
Parents	300	120	143	37				0.57	0.638	0.362	
Probands	150	55	66	29				0.25	0.587	0.413	
rs504348		**GG**	**GC**	**CC**					**G**	**C**	
Parents	300	293	7	0				0.84	0.988	0.012	
Probands	150	146	4	0				0.87	0.987	0.013	
rs717620		**TT**	**TC**	**CC**					**T**	**C**	
Parents	300	15	91	194				0.32	0.202	0.798	
Probands	150	5	55	90				0.33	0.217	0.783	
rs2273697		**AA**	**AG**	**GG**					**A**	**G**	
Parents	300	4	56	240				0.72	0.107	0.893	
Probands	150	1	24	125				0.89	0.087	0.913	
rs2231142		**AA**	**AC**	**CC**					**A**	**C**	
Parents	300	20	132	148				0.19	0.287	0.713	
Probands	150	13	56	81				0.46	0.273	0.727	
rs2231137		**AA**	**AG**	**GG**					**A**	**G**	
Parents	300	33	125	142				0.49	0.318	0.681	
Probands	150	16	72	62				0.47	0.347	0.653	
rs2054576		**CC**	**CT**	**TT**					**C**	**T**	
Parents	300	17	110	173				0.93	0.240	0.760	
Probands	150	9	50	91				0.36	0.227	0.773	

Table 12 Transmission disequilibrium test in oral clefts cases

Gene/Marker	Marker Allele	n1	n2	T/NT	P-value for allele-wise TDT	Odds ratio	95% CI
ABCB1: 12/1236 (rs1128503)	T>C	150	108	**T**: 92/45 **C**: 45/92	0.000051	2.044	1.416 - 2.989
ABCB1: 21/2677 (rs2032582)	G>T, A	150	111	**G**: 78/91 **T**: 96/64 **A**: 23/42	0.008592	1.500 (For T allele)	1.082 - 2.091
ABCB1: 26/3435 (rs1045642)	C>T	150	110	**C**: 56/87 **T**: 87/56	0.009277	1.554	1.098 - 2.214
ABCC1: 5'FR/-260 (rs504348)	G>C	150	7	**G**: 3/4 **C**: 4/3	0.704992	1.333	0.226 - 9.102
ABCC2: e1/-24 (rs717620)	C>T	150	76	**C**: 41/50 **T**: 50/41	0.345100	1.244	0.808 - 1.925
ABCC2: e10/1249 (rs2273697)	G>A	150	53	**G**: 34/22 **A**: 22/34	0.107503	1.545	0.878 - 2.774
ABCG2: e5/421 (rs2231142)	C>A	150	110	**C**: 70/62 **A**: 62/70	0.486130	1.129	0.791 - 1.616
ABCG2: e2/34 (rs2231137)	G>A	150	105	**G**: 54/71 **A**: 71/54	0.127852	1.315	0.910 - 1.909
ABCG2: I9/-357 (rs2054576)	T>C	150	91	**T**: 59/51 **C**: 51/59	0.445434	1.157	0.782 - 1.717

n1: Number of family samples which were successfully genotyped.
n2: Number families with at least one heterozygote parent.
T: Transmitted
NT: non-transmitted

3.1.2 Statistical analysis on the genotype data of cases and controls

The genotype distribution of all polymorphisms investigated was in Hardy-Weinberg equilibrium (χ^2<3.84, P>0.05) in oral cleft cases, their mothers and controls.

Results of the Fisher's exact test and the Chi-square analysis revealed no significant differences in the alleles' frequency of the target SNPs between Chinese mothers of oral cleft children and the controls (Table 13). However, in another comparison between the Chinese oral cleft cases and the controls, interestingly a significant difference revealed in the alleles' frequency of the *ABCB1* SNP e12/1236 (rs1128503) (OR = 1.60, P = 0.0046) and it was remained significant even after Bonferroni correction for multiple testing. In this analysis, no significant differences found in the alleles' frequencies of other examined SNPs between the cases and controls. The results are given in details in Table 14.

In line with HapMap reports, the *ABCC1* SNP at position 5'FR/G-260C exhibited very low minor allele frequency in our samples; however, this SNP was genotyped to test for penetration.

Table 13 Genotype and allele frequencies of SNPs and association analysis of each SNP in controls and mothers of oral cleft children

Polymorphism	No	Genotype						HWE (p)	Allele frequencies			P-value for allele-wise Fisher's exact/ Chi-square test	OR	95% CI
rs1128503		**TT**	**TC**	**CC**					**T**	**C**				
Controls	189	78	80	31				0.18	236 (0.624)	142 (0.376)		0.4186	0.95	0.69 - 1.32
Mothers	129	53	58	18				0.74	164 (0.636)	94 (0.364)				
rs2032582		**AA**	**AG**	**AT**	**GG**	**GT**	**TT**		**A**	**G**	**T**			
Controls	189	2	25	11	42	72	37	0.22	40 (0.106)	181 (0.479)	157 (0.415)	0.1889	1.35	0.05 - 7.38
Mothers	129	2	20	9	33	50	15	0.72	33 (0.128)	136 (0.527)	89 (0.345)			
rs1045642		**CC**	**CT**	**TT**					**C**	**T**				
Controls	189	71	86	32				0.46	228 (0.603)	150 (0.397)		0.0321	1.39	0.99 - 1.93
Mothers	129	61	53	15				0.51	175 (0.678)	83 (0.321)				
rs504348		**GG**	**GC**	**CC**					**G**	**C**				
Controls	189	186	3	0				0.91	375 (0.992)	3 (0.008)		0.4651	0.49	0.05 - 4.70
Mothers	129	128	1	0				0.97	257 (0.996)	1 (0.004)				
rs717620		**TT**	**TC**	**CC**					**T**	**C**				
Controls	189	6	52	131				0.76	64 (0.169)	314 (0.831)		0.1763	0.81	0.54 - 1.21
Mothers	129	7	38	84				0.36	52 (0.202)	206 (0.798)				
rs2273697		**AA**	**AG**	**GG**					**A**	**G**				
Controls	189	0	36	153				0.15	36 (0.095)	342 (0.905)		0.2105	1.32	0.74 - 2.36
Mothers	129	0	19	110				0.37	19 (0.074)	239 (0.926)				
rs2231142		**AA**	**AC**	**CC**					**A**	**C**				
Controls	189	11	83	95				0.19	105 (0.278)	273 (0.722)		0.5207	0.99	0.70 - 1.41
Mothers	129	11	50	68				0.68	72 (0.279)	186 (0.721)				
rs2231137		**AA**	**AG**	**GG**					**A**	**G**				
Controls	189	18	92	79				0.23	128 (0.339)	250 (0.661)		0.4274	0.96	0.69 - 1.33
Mothers	129	15	60	54				0.79	90 (0.349)	168 (0.651)				
rs2054576		**CC**	**CT**	**TT**					**C**	**T**				
Controls	189	6	68	115				1.16	80 (0.212)	298 (0.788)		0.1632	1.23	0.84 - 1.79
Mothers	129	10	44	75				0.95	64 (0.248)	194 (0.752)				

Table 14 Genotype and allele frequencies of SNPs and association analysis of each SNP in controls and oral cleft sample

Polymorphism	No	Genotype						HWE (p)	Allele frequencies			P-value for allele-wise Fisher's exact/ Chi-square test	OR	95% CI
rs1128503		**TT**	**TC**	**CC**					**T**		**C**			
Controls	189	78	80	31				0.18	236 (0.624)		142 (0.376)	0.0046	1.60	1.13 - 2.26
Oral clefts	128	68	50	10				0.85	186 (0.727)		70 (0.273)			
rs2032582		**AA**	**AG**	**AT**	**GG**	**GT**	**TT**		**A**	**G**	**T**			
Controls	189	2	25	11	42	72	37	0.22	40 (0.106)	181 (0.479)	157 (0.415)	0.8622	0.94	0.05 - 7.38
Oral clefts	128	0	15	9	28	51	25	0.46	24 (0.094)	122 (0.477)	110 (0.430)			
rs1045642		**CC**	**CT**	**TT**					**C**		**T**			
Controls	189	71	86	32				0.46	228 (0.603)		150 (0.397)	0.4770	0.98	0.70 - 1.35
Oral clefts	128	49	55	24				0.23	153 (0.598)		103 (0.402)			
rs504348		**GG**	**GC**	**CC**					**G**		**C**			
Controls	189	186	3	0				0.91	375 (0.992)		3 (0.008)	0.6765	1.016	0.17 - 6.12
Oral clefts	128	126	2	0				0.93	254 (0.992)		2 (0.008)			
rs717620		**TT**	**TC**	**CC**					**T**		**C**			
Controls	189	6	52	131				0.76	64 (0.169)		314 (0.831)	0.1121	1.31	1.31 - 0.88
Oral clefts	128	3	48	77				0.15	54 (0.210)		202 (0.789)			
rs2273697		**AA**	**AG**	**GG**					**A**		**G**			
Controls	189	0	36	153				0.15	36 (0.095)		342 (0.905)	0.4009	0.89	0.51 - 1.56
Oral clefts	128	1	20	107				0.95	22 (0.086)		234 (0.914)			
rs2231142		**AA**	**AC**	**CC**					**A**		**C**			
Controls	189	11	83	95				0.19	105 (0.278)		273 (0.722)	0.3319	1.01	0.77 - 1.56
Oral clefts	128	13	50	65				0.47	76 (0.297)		180 (0.703)			
rs2231137		**AA**	**AG**	**GG**					**A**		**G**			
Controls	189	18	92	79				0.23	128 (0.339)		250 (0.661)	0.5069	0.99	0.71 - 1.38
Oral clefts	128	14	58	56				0.86	86 (0.336)		170 (0.664)			
rs2054576		**CC**	**CT**	**TT**					**C**		**T**			
Controls	189	6	68	115				1.16	80 (0.212)		298 (0.788)	0.1505	1.24	0.85 - 1.81
Oral clefts	128	9	46	73				0.64	64 (0.250)		192 (0.750)			

3.1.3 Joint analysis of TDT and Case-control results

Table 15 details the results of a joint analysis on the TDT and case-control results for the *ABCB1* SNP e12/1236 that found to be significantly associated with non-syndromic oral clefts in the family and the case-control based studies.

The analysis disclosed that the odds ratios were homogenous in the two family based and case-control studies (P*>0.05) and the estimate of the combined odds ratio was calculated by 1.79, which was highly significant (P-value <0.0001).

Table 15 The results of joint analysis of the TDT and case-control studies

	Case-Control study		**TDT study**		**Combined results**			
***ABCB1* SNP**	**OR**	**95% CI**	**OR**	**95% CI**	**ORc**	**95% CI**	**P-value**	**P***
rs1128503 (e12/1236)	1.58	1.12 - 2.23	2.04	1.42 - 2.99	1.79	1.38 - 2.30	<0.0001	0.32

P*: P-value for test of homogeneity of odds ratios.

3.2 Discussion

The effect of most teratogens on fetal development is dependent on the fetal exposure. Therefore, during fetal development modulation of maternal exposure to environmental factors as well as the modulation of fetal exposure to the potentially toxic xenobiotics digested by the mother is vital. As ABC transporter proteins are thought to be involved in such modulation, this study aimed to investigate the association of nine potentially functional SNPs within the four ABC transporter genes

known to be expressed in maternal barrier organs, such as intestine, kidney, liver, lung, and the fetal barrier organ i.e. placenta with non-syndromic oral clefts.

To best of our knowledge there is no evidence to support that a spontaneous mutation in these human genes can increase the risk of orofacial clefting as previously demonstrated in mice by Lankas et al. (1998). The current study was among the first to examine several major ABC genes polymorphisms in children born with orofacial clefts.

Our present study of 150 nuclear families showed that the *ABCB1* SNPs e12/1236 is significantly associated with non-syndromic oral clefts and the two other *ABCB1* SNPs e21/2677 and e26/3435 have a nominal association. Interestingly, this finding was validated by the case-control study of the oral clefts children despite the fact that the power of our case-control study is estimated to be less than 50% for SNP e12/1236 and the sample size used. Moreover, the joint analysis on the family based and case-control studies could contrast the results of the two studies and revealed that there is homogeneity in the strength of the association found.

One might envision a number of possible explanations for the observed association. Nonetheless, to have a better explanation, additional analysis was performed on distribution of homozygosity for the target SNPs alleles in Chinese oral cleft families (Table 10), which showed the maternal genotypes had no synergistic effect when both mother and child were homozygote for the mutant SNPs alleles. This was further confirmed when genotypes and alleles frequencies of the target SNPs compared between the Chinese mothers of oral cleft children against controls that showed no significant difference between the two groups. This could support the hypothesis that the *ABCB1* genotype of the fetus and not the mother is a critical factor in determining

the disease risk. On the other hand, in case of less ABCB1/P-gp expression in the maternal barrier organs, the placental ABCB1 whose expression is influenced by the fetal genotype can serve a compensatory role in protecting the fetus from potentially toxicants passed the maternal barriers. In contrast, fetal *ABCB1* polymorphisms may increase the risk of orofacial clefting by altering the P-gp expression or activity in the placenta.

The obtained results suggest that the *ABCB1* SNPs e21/2677 and e26/3435 in the absence of multiple test correction (MTC) and the SNP e12/1236 even after MTC can mediate the diseases risk. As discussed in chapter one, previous studies have revealed the functionality of the SNP e12/1236 in various cells but not yet in the placenta. However, the studies have disclosed the effect of *ABCB1* polymorphisms at e21/2677 and e26/3435 on P-gp expression in term placentas. Given the placental P-gp expression tends to decrease in late pregnancy, it may be necessary to do further evaluation in preterm along with the term placentas to clarify the influence of the SNP C1236T on P-gp expression/activity in this organ. The SNP C1236T is a non-synonymous SNP, but Aird et al. (2007) have showed in 3T3 isogenic fibroblasts, mutation at position 1236 can significantly alter *ABCB1* expression, regardless of additional mutations at positions 2677 and 3435.

Additionally, the allelic-wise analysis in our family based and case-control study disclosed a preferential transmission for the T allele of this SNP compared to the C allele. This was in line with the report of Vaclavikovaa et al. (2008) that the T variant of the SNP C1236T can significantly alter *ABCB1* expression in breast tumors. Assuming the SNP e12/1236T can result in less P-gp expression in early placenta at the time of lip and palate formation, the association found for this polymorphism

favors the hypothesis that fetal genotypes harboring the T allele of the SNP C1236T might be more vulnerable to orofacial clefts especially in case of maternal smoking or pharmacotherapy. It is also possible that the SNP C1236T is not a causal polymorphism but is merely in strong linkage disequilibrium with an unobserved causal SNP.

The allelic-wise SNP analysis also revealed that the T allele of SNP 2677 has significantly higher transmission to the oral cleft probands over the A and G alleles, although the A allele also showed a higher transmission over the wild type G allele in the absence of Bonferroni correction. Moreover, the T allele of SNP 3435 was found to have higher transmission to the affected offsprings over the C allele. This findings are consistent with the previous studies in term placentas that reported the *ABCB1* SNPs 2677T/A and 3435T were found to be associated with less placental P-gp expression (Tanabe et al. 2001; Hitzl et al. 2004; Sherif et al 2007). As discussed in chapter one, studies on kidney and heart tissues have reported higher level of ABCB1 mRNA expression for the carries of T and A alleles of SNP 2677 compared to the G carriers. However, the regulation of P-gp expression may significantly differ from tissue to tissue, explaining the conflicting reports for the placenta. Moreover, linkage studies in Chinese, Malays, and Indians have disclosed that the T alleles of three SNPs e12/1236, e26/3435, e21/2677 are frequently linked together. Thus, the observed preference in transmission of the T alleles of SNPs 3435 and 2677 might be due to their linkage to the SNP 1236T that found to be significantly associated with oral clefting.

Taken together, further studies need to clarify whether fetal *ABCB1* genetics determines the risk of orofacial malformations in maternal smoking and after

unintended or intended maternal drug treatment. It is known that the *ABCB1* expression could be related to both genetic polymorphism and environmental factors, such as drugs and smoking. However, in an experiment by Kolwankar et al. (2005) no significant difference was found in placental P-gp activity or expression between smokers and nonsmokers. Thus, in the current discussion, the genetic polymorphism was considered as the main determinant factor for P-gp expression in placenta regardless of maternal history for smoking and drug treatment in pregnancy.

However, it is still possible that in spite of a wild type *ABCB1* genotype and high level of placental P-gp expression, fetus exposes to potentially toxic xenobiotics. Because the concurrent exposure to multiple Pg-p substrates or substrates that are Pg-p inhibitor can also impair the placental P-gp activity and increase the disease risk. A limitation of our study was the fact that we were not able to study the relationship between the *ABCB1* gene polymorphisms and maternal histories of smoking and pharmacotherapy during pregnancy for the mothers participated in the study. Therefore, it remains open whether the findings of this study really reflect the role of the placental *ABCB1* polymorphisms in increasing the risk of orofacial clefting.

As discussed in chapter one, ABCB1 in the early stage of gestation, but in late gestation ABCC1 and ABCC2 and in mid gestation ABCG2 were found to be highly expressed in placenta. Given that the formation of lip and palate completes by the first trimester of pregnancy, the role of placental ABCB1/P-gp seems to be more crucial than the mentioned ABC transporters in giving protection to the fetus against harmful environmental factors. Consistent with this issue in this study, *ABCC1*, *ABCC2* and *ABCG2* polymorphisms found not to be associated with oral clefting ($P>0.05$). However, it should be confirmed through a study with larger sample size or in

ethnicities, which exhibit higher minor allele frequency (MAF) for the examined SNPs. For instance, *ABCC1* SNP 5'FR/G-260C exhibited a very low MAF in our study population, thus investigation of this variant is suggested in a similar study on other ethnicities, such as Caucasians that show higher MAF for the SNP.

Strengths of this study include the family based study that is robust against population substructure and confounding effects causing by population admixture and the case-control study conducted in parallel with the family association study. It should also note that haplotype and linkage analysis were not performed for the polymorphisms that are in the same gene, as the aim of this study was to evaluate the role of the potentially functional SNPs as candidate disease susceptibility alleles and such analysis are irrelevant to this study.

3.3 Conclusion

ABCB1 polymorphisms at positions 2766, and 3435 appeared to be associated with susceptibility to orofacial clefting in the absence of multiple testing correction and the SNP C1236T was significantly associated even when type I error reduction was performed. However, no significant difference observed in distribution of maternal target SNPs alleles and genotypes in association with oral clefting in the offsprings. Thus, because of the fetal derivation of placental tissues, the P-gp genotype of the fetus is a critical factor in determining the degree of fetal exposure to substrates of this transport protein as well as the degree of the drugs teratogenicity. This also supports the hypothesis that the placental ABCB1/P-gp can influence the barrier function of this organ against teratogens.

Moreover, depending on the developmental toxicity profile of the Pg-p substrates/inhibitors, inhibition of the placental Pg-p activity may have deleterious effects on human fetal development. Thus, screening compounds or natural products to which women may be exposed during pregnancy for effects on placental Pg-p function or expression may be useful in protection of fetus, especially on the ground of *ABCB1* polymorphisms at e12/1236, e21/2677, and e26/3435. This research also represents the importance of risk assessment for drug prescriptions in pregnancy and choice of therapy for a safer clinical practice.

REFERENCES

Aird, Thomson, et al. (2008). "ABCB1 genetic polymorphism influences the pharmacology of the new pyrrolobenzodiazepine derivative SJG-136." Pharmacogenomics J **8**(4): 289-96.

Ameyaw, Regateiro, et al. (2001). "MDR1 pharmacogenetics: frequency of the C3435T mutation in exon 26 is significantly influenced by ethnicity." Pharmacogenetics **11**(3): 217-21.

Atkinson, Greenwood, et al. (2003). "Role of MDR1 and MRP1 in trophoblast cells, elucidated using retroviral gene transfer." Am J Physiol Cell Physiol **285**(3): C584-91.

Atkinson, Brice-Bennett, et al. (2007). "Antiepileptic medication during pregnancy: does fetal genotype affect outcome?" Pediatr Res **62**(2): 120-7.

Backstrom, Taipalensuu, et al. (2003). "Genetic variation in the ATP-binding cassette transporter gene ABCG2 (BCRP) in a Swedish population." Eur J Pharm Sci **18**(5): 359-64.

Balram, Sharma, et al. (2003). "Frequency of C3435T single nucleotide MDR1 genetic polymorphism in an Asian population: phenotypic-genotypic correlates." Br J Clin Pharmacol **56**(1): 78-83.

Bernal, Sinues, et al. (2003). "Frequency distribution of C3435T mutation in exon 26 of the MDR1 gene in a Spanish population." Ther Drug Monit **25**(1): 107-11.

Borst, Evers, et al. (1999). "The multidrug resistance protein family." Biochim Biophys Acta **1461**(2): 347-57.

Borst, Evers, et al. (2000). "A family of drug transporters: the multidrug resistance-associated proteins." J Natl Cancer Inst **92**(16): 1295-302.

Cascorbi, Gerloff, et al. (2001). "Frequency of single nucleotide polymorphisms in the P-glycoprotein drug transporter MDR1 gene in white subjects." Clin Pharmacol Ther **69**(3): 169-74.

Ceckova, Libra, et al. (2006). "Expression and functional activity of breast cancer resistance protein (BCRP, ABCG2) transporter in the human choriocarcinoma cell line BeWo." Clin Exp Pharmacol Physiol **33**(1-2): 58-65.

Chowbay, Cumaraswamy, et al. (2003). "Genetic polymorphisms in MDR1 and CYP3A4 genes in Asians and the influence of MDR1 haplotypes on cyclosporin disposition in heart transplant recipients." Pharmacogenetics **13**(2): 89-95.

Cole, Bhardwaj, et al. (1992). "Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line." Science **258**(5088): 1650-4.

Cole and Deeley (1998). "Multidrug resistance mediated by the ATP-binding cassette transporter protein MRP." Bioessays **20**(11): 931-40.

Conseil, Deeley, et al. (2005). "Polymorphisms of MRP1 (ABCC1) and related ATP-dependent drug transporters." Pharmacogenet Genomics **15**(8): 523-33.

de Jong, Marsh, et al. (2004). "ABCG2 pharmacogenetics: ethnic differences in allele frequency and assessment of influence on irinotecan disposition." Clin Cancer Res **10**(17): 5889-94.

Dean, Rzhetsky, et al. (2001). "The human ATP-binding cassette (ABC) transporter superfamily." Genome Res **11**(7): 1156-66.

Drozdzik, Bialecka, et al. (2003). "Polymorphism in the P-glycoprotein drug transporter MDR1 gene: a possible link between environmental and genetic factors in Parkinson's disease." Pharmacogenetics **13**(5): 259-63.

Eisenblatter and Galla (2002). "A new multidrug resistance protein at the blood-brain barrier." Biochem Biophys Res Commun **293**(4): 1273-8.

Furuno, Landi, et al. (2002). "Expression polymorphism of the blood-brain barrier component P-glycoprotein (MDR1) in relation to Parkinson's disease." Pharmacogenetics **12**(7): 529-34.

Gaikovitch, Cascorbi, et al. (2003). "Polymorphisms of drug-metabolizing enzymes CYP2C9, CYP2C19, CYP2D6, CYP1A1, NAT2 and of P-glycoprotein in a Russian population." Eur J Clin Pharmacol **59**(4): 303-12.

Gauderman (2002). "Sample size requirements for matched case-control studies of gene-environment interaction." Stat Med **21**(1): 35-50.

Gennuso, Fernetti, et al. (2004). "Bilirubin protects astrocytes from its own toxicity by inducing up-regulation and translocation of multidrug resistance-associated protein 1 (Mrp1)." Proc Natl Acad Sci U S A **101**(8): 2470-5.

Gerloff, Schaefer, et al. (2002). "MDR1 genotypes do not influence the absorption of a single oral dose of 1 mg digoxin in healthy white males." Br J Clin Pharmacol **54**(6): 610-6.

Gil, Saura, et al. (2005). "P-glycoprotein expression of the human placenta during pregnancy." Placenta **26**(2-3): 268-70.

Gottesman (2002). "Mechanisms of cancer drug resistance." Annu Rev Med **53**: 615-27.

Haenisch, Zimmermann, et al. (2007). "Influence of polymorphisms of ABCB1 and ABCC2 on mRNA and protein expression in normal and cancerous kidney cortex." Pharmacogenomics J **7**(1): 56-65.

Hipfner, Deeley, et al. (1999). "Structural, mechanistic and clinical aspects of MRP1." Biochim Biophys Acta **1461**(2): 359-76.

Hitzl, Schaeffeler, et al. (2004). "Variable expression of P-glycoprotein in the human placenta and its association with mutations of the multidrug resistance 1 gene (MDR1, ABCB1)." Pharmacogenetics **14**(5): 309-18.

Hoffmeyer, Burk, et al. (2000). "Functional polymorphisms of the human multidrug-resistance gene: multiple sequence variations and correlation of one allele with P-glycoprotein expression and activity in vivo." Proc Natl Acad Sci U S A **97**(7): 3473-8.

Hollenstein, Dawson, et al. (2007). "Structure and mechanism of ABC transporter proteins." Curr Opin Struct Biol **17**(4): 412-8.

Hooijberg, Jansen, et al. (2004). "Folate concentration dependent transport activity of the Multidrug Resistance Protein 1 (ABCC1)." Biochem Pharmacol **67**(8): 1541-8.

Horinouchi, Sakaeda, et al. (2002). "Significant genetic linkage of MDR1 polymorphisms at positions 3435 and 2677: functional relevance to pharmacokinetics of digoxin." Pharm Res **19**(10): 1581-5.

Ifergan, Shafran, et al. (2004). "Folate deprivation results in the loss of breast cancer resistance protein (BCRP/ABCG2) expression. A role for BCRP in cellular folate homeostasis." J Biol Chem **279**(24): 25527-34.

Imai, Nakane, et al. (2002). "C421A polymorphism in the human breast cancer resistance protein gene is associated with low expression of Q141K protein and low-level drug resistance." Mol Cancer Ther **1**(8): 611-6.

Ito, Suzuki, et al. (2005). "Apical/basolateral surface expression of drug transporters and its role in vectorial drug transport." Pharm Res **22**(10): 1559-77.

Johne, Kopke, et al. (2002). "Modulation of steady-state kinetics of digoxin by haplotypes of the P-glycoprotein MDR1 gene." Clin Pharmacol Ther **72**(5): 584-94.

Kage, Tsukahara, et al. (2002). "Dominant-negative inhibition of breast cancer resistance protein as drug efflux pump through the inhibition of S-S dependent homodimerization." Int J Cancer **97**(5): 626-30.

Kazeem and Farrall (2005). "Integrating case-control and TDT studies." Ann Hum Genet **69**(Pt 3): 329-35.

Kim, Leake, et al. (2001). "Identification of functionally variant MDR1 alleles among European Americans and African Americans." Clin Pharmacol Ther **70**(2): 189-99.

Kimchi-Sarfaty, Oh, et al. (2007). "A "silent" polymorphism in the MDR1 gene changes substrate specificity." Science **315**(5811): 525-8.

Kobayashi, Ieiri, et al. (2005). "Functional assessment of ABCG2 (BCRP) gene polymorphisms to protein expression in human placenta." Drug Metab Dispos **33**(1): 94-101.

Kolwankar, Glover, et al. (2005). "Expression and function of ABCB1 and ABCG2 in human placental tissue." Drug Metab Dispos **33**(4): 524-9.

Kondo, Suzuki, et al. (2004). "Functional analysis of SNPs variants of BCRP/ABCG2." Pharm Res **21**(10): 1895-903.

Krishnamurthy and Schuetz (2006). "Role of ABCG2/BCRP in biology and medicine." Annu Rev Pharmacol Toxicol **46**: 381-410.

Lankas, Wise, et al. (1998). "Placental P-glycoprotein deficiency enhances susceptibility to chemically induced birth defects in mice." Reprod Toxicol **12**(4): 457-63.

Leslie, Deeley, et al. (2001). "Toxicological relevance of the multidrug resistance protein 1, MRP1 (ABCC1) and related transporters." Toxicology **167**(1): 3-23.

Maliepaard, Scheffer, et al. (2001). "Subcellular localization and distribution of the breast cancer resistance protein transporter in normal human tissues." Cancer Res **61**(8): 3458-64.

Marzolini, Paus, et al. (2004). "Polymorphisms in human MDR1 (P-glycoprotein): recent advances and clinical relevance." Clin Pharmacol Ther **75**(1): 13-33.

Mathias, Hitti, et al. (2005). "P-glycoprotein and breast cancer resistance protein expression in human placentae of various gestational ages." Am J Physiol Regul Integr Comp Physiol **289**(4): R963-9.

Mathijssen, Marsh, et al. (2003). "Irinotecan pathway genotype analysis to predict pharmacokinetics." Clin Cancer Res **9**(9): 3246-53.

May, Minarikova, et al. (2008). "Role of the multidrug transporter proteins ABCB1 and ABCC2 in the diaplacental transport of talinolol in the term human placenta." Drug Metab Dispos **36**(4): 740-4.

Meissner, Jedlitschky, et al. (2004). "Modulation of multidrug resistance P-glycoprotein 1 (ABCB1) expression in human heart by hereditary polymorphisms." Pharmacogenetics **14**(6): 381-5.

Meyer zu Schwabedissen, Jedlitschky, et al. (2005). "Variable expression of MRP2 (ABCC2) in human placenta: influence of gestational age and cellular differentiation." Drug Metab Dispos **33**(7): 896-904.

Meyer zu Schwabedissen, Grube, et al. (2006). "Epidermal growth factor-mediated activation of the map kinase cascade results in altered expression and function of ABCG2 (BCRP)." Drug Metab Dispos **34**(4): 524-33.

Mizuarai, Aozasa, et al. (2004). "Single nucleotide polymorphisms result in impaired membrane localization and reduced atpase activity in multidrug transporter ABCG2." Int J Cancer **109**(2): 238-46.

Morisaki, Robey, et al. (2005). "Single nucleotide polymorphisms modify the transporter activity of ABCG2." Cancer Chemother Pharmacol **56**(2): 161-72.

Moriya, Nakamura, et al. (2002). "Effects of polymorphisms of MDR1, MRP1, and MRP2 genes on their mRNA expression levels in duodenal enterocytes of healthy Japanese subjects." Biol Pharm Bull **25**(10): 1356-9.

Nagashige, Ushigome, et al. (2003). "Basal membrane localization of MRP1 in human placental trophoblast." Placenta **24**(10): 951-8.

Nakamura, Sakaeda, et al. (2002). "Effect of the mutation (C3435T) at exon 26 of the MDR1 gene on expression level of MDR1 messenger ribonucleic acid in duodenal enterocytes of healthy Japanese subjects." Clin Pharmacol Ther **71**(4): 297-303.

Ozawa, Soyama, et al. (2004). "Ethnic differences in genetic polymorphisms of CYP2D6, CYP2C19, CYP3As and MDR1/ABCB1." Drug Metab Pharmacokinet **19**(2): 83-95.

Pascolo, Fernetti, et al. (2003). "Effects of maturation on RNA transcription and protein expression of four MRP genes in human placenta and in BeWo cells." Biochem Biophys Res Commun **303**(1): 259-65.

Pohl, Devaux, et al. (2005). "Function of prokaryotic and eukaryotic ABC proteins in lipid transport." Biochim Biophys Acta **1733**(1): 29-52.

Roberts, Joyce, et al. (2002). "A common P-glycoprotein polymorphism is associated with nortriptyline-induced postural hypotension in patients treated for major depression." Pharmacogenomics J **2**(3): 191-6.

Sakaeda, Nakamura, et al. (2001). "MDR1 genotype-related pharmacokinetics of digoxin after single oral administration in healthy Japanese subjects." Pharm Res **18**(10): 1400-4.

Schaeffeler, Eichelbaum, et al. (2001). "Frequency of C3435T polymorphism of MDR1 gene in African people." Lancet **358**(9279): 383-4.

Sham and Curtis (1995). "An extended transmission/disequilibrium test (TDT) for multi-allele marker loci." Ann Hum Genet **59**(Pt 3): 323-36.

Sherif, Tatiana, et al. (2007). "Effect of the G2677A/T polymorphism in the multidrug resistance 1 gene (MDR1, ABCB1) on P-glycoprotein (P-gp) expression in human placenta." Am J Obs Gyn **197**(6): S172.

Siddiqui, Kerb, et al. (2003). "Association of multidrug resistance in epilepsy with a polymorphism in the drug-transporter gene ABCB1." N Engl J Med **348**(15): 1442-8.

Siegmund, Ludwig, et al. (2002). "The effects of the human MDR1 genotype on the expression of duodenal P-glycoprotein and disposition of the probe drug talinolol." Clin Pharmacol Ther **72**(5): 572-83.

Smit, Huisman, et al. (1999). "Absence or pharmacological blocking of placental P-glycoprotein profoundly increases fetal drug exposure." J Clin Invest **104**(10): 1441-7.

Song, Lamba, et al. (2006). "G2677T and C3435T genotype and haplotype are associated with hepatic ABCB1 (MDR1) expression." J Clin Pharmacol **46**(3): 373-9.

St-Pierre, Serrano, et al. (2000). "Expression of members of the multidrug resistance protein family in human term placenta." Am J Physiol Regul Integr Comp Physiol **279**(4): R1495-503.

Sun, Kingdom, et al. (2006). "Expression of the multidrug resistance P-glycoprotein, (ABCB1 glycoprotein) in the human placenta decreases with advancing gestation." Placenta **27**(6-7): 602-9.

Tamura, Wakabayashi, et al. (2006). "Genetic polymorphisms of human ABC transporter ABCG2: development of the standard method for functional validation of SNPs by using the Flp recombinase system." J Exp Ther Oncol **6**(1): 1-11.

Tamura, Wakabayashi, et al. (2007). "Re-evaluation and functional classification of non-synonymous single nucleotide polymorphisms of the human ATP-binding cassette transporter ABCG2." Cancer Sci **98**(2): 231-9.

Tanabe, Ieiri, et al. (2001). "Expression of P-glycoprotein in human placenta: relation to genetic polymorphism of the multidrug resistance (MDR)-1 gene." J Pharmacol Exp Ther **297**(3): 1137-43.

Tang, Ngoi, et al. (2002). "Distinct haplotype profiles and strong linkage disequilibrium at the MDR1 multidrug transporter gene locus in three ethnic Asian populations." Pharmacogenetics **12**(6): 437-50.

Vaclavikova, Nordgard, et al. (2008). "Single nucleotide polymorphisms in the multidrug resistance gene 1 (ABCB1): effects on its expression and clinicopathological characteristics in breast cancer patients." Pharmacogenet Genomics **18**(3): 263-73.

Wang*, Johnson, et al. (2005). "Multidrug resistance polypeptide 1 (MDR1, ABCB1) variant 3435C>T affects mRNA stability." Pharmacogenet Genomics **15**(10): 693-704.

Wang, Wang, et al. (2005). "A functional polymorphism within the MRP1 gene locus identified through its genomic signature of positive selection." Hum Mol Genet **14**(14): 2075-87.

Wang, Wu, et al. (2006). "Expression of the breast cancer resistance protein (Bcrp1/Abcg2) in tissues from pregnant mice: effects of pregnancy and correlations with nuclear receptors." Am J Physiol Endocrinol Metab **291**(6): E1295-304.

Wang, Wang, et al. (2007). "Signatures of recent positive selection at the ATP-binding cassette drug transporter superfamily gene loci." Hum Mol Genet **16**(11): 1367-80.

Yanase, Tsukahara, et al. (2006). "Functional SNPs of the breast cancer resistance protein-therapeutic effects and inhibitor development." Cancer Lett **234**(1): 73-80.

Yeo, Van Nostrand, et al. (2007). "Discovery and analysis of evolutionarily conserved intronic splicing regulatory elements." PLoS Genet **3**(5): e85.

Zhou, Schuetz, et al. (2001). "The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype." Nat Med **7**(9): 1028-34.

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