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Studies of γ -Aminobutyric Acid Type A Receptor $\beta 3$ (GABRB3) and Glutamic Acid Decarboxylase 67 (GAD67) with Oral Clefts

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Dear Editor:

CL/P is a common birth defect that stems from multiple genetic and environmental factors, which cause disruption in normal lip and/or palate formation. Non-syndromic cleft lip and/or cleft palate affects 1-2/1000 individuals born with rates varying between ethnic groups [Murray, 2002]. γ -Aminobutyric acid type A receptors (GABA_A-Rs) are major inhibitory neurotransmitters in the central nervous system, coded for by the gene GABRB3. GABA acid, which is synthesized from glutamate by the protein glutamic acid decarboxylase (GAD), binds with GABA_A-Rs to facilitate the inhibitory process. GAD exists in two isoforms: GAD65 and GAD67. Mouse models have shown that decreased expression of either GABRB3 or GAD67 result in cleft lip with or without cleft palate (CL/P). In one study, knockout mice for GABRB3 had a high incidence of cleft palate [Homanics et al., 1997]. In another study looking at the effects of GAD67, mice devoid of the gene showed similar results with high mortality rates due to severe clefting [Asada et al., 1997]. Genotyping of either gene in humans has also suggested a role for the genes in human facial morphogenesis as well. Significant linkage disequilibrium (LD) between alleles in GABRB3 and CL/P was obtained when tested on an Italian population [Scapoli et al., 2002]. Similarly for GAD67, significant LD results were obtained on a Japanese population [Kanno et al., 2004] and it has been hypothesized it may play a role in Pierre-Robin sequence also [Jakobsen et al, 2006]. In this study we further examined the connection between both the GABRB3 and GAD67 genes and non-syndromic clefting.

Blood samples from the Philippines were collected under the auspices of Operation Smile at one of four locations (Cavite, Kalibo, Cebu, and Negros) from affected cases and parents whenever available. The samples came from 307 CL/P and CPO patients [Murray et al., 1997]. Blood samples from Iowa were collected through the Iowa Birth Defects Registry. The samples came from 205 CL/P and CPO children and their parents and were collected between January 1, 1987 and December 31, 1991 [Romitti et al., 1998]. All samples were collected with signed consent and had local and/or University of Iowa IRB approval. Subjects with clefts were screened for the existence of any associated anomalies or syndromes and only those with isolated forms of CL/P and CPO were used in this study.

Genotyping was performed on nine single nucleotide polymorphisms on the GABRB3 gene: rs1426217; rs1593610; rs2059574; rs4477673; rs4572353; rs657575; rs6576618; rs890317;

and rs981778. On the GAD67 gene two single nucleotide polymorphisms were genotyped in each population: in Filipinos rs3749034 and rs2058725; in Iowans rs3749034 and rs2177433. These polymorphisms were selected based on their allele frequencies and the haplotype structure of the genes from the International HapMap Project (www.hapmap.org). In the case of the GAD67 rs3749034, this marker was chosen because it was included in the haplotype that was associated with clefts in Japanese [Kanno et al., 2004]. Genotyping was done on a GeneAmp 7900 Sequence Detection System (ABI Prism 7900HT, Applied Biosystems) using TaqMan technology [Ranade et al. 2001]. Assays were obtained from Applied Biosystems through either their Assay-by-Design or Assay-by-Demand service.

Data was analyzed according to the following subphenotypes: all clefts (ALL), cleft lip only (CPO), cleft lip with cleft palate (CLP), cleft palate only (CPO), cleft lip with or without cleft palate (CL/P), and cleft lip with cleft palate and cleft palate only (CLP + CPO). CPO samples were available only for the Iowa population. The program FBAT (Family Based Association Test) was used for linkage disequilibrium analysis on both the Iowa and Filipino populations [Horvath et al. 2001]. We tested for single marker associations, as well as for haplotype associations. Haplotypes were chosen based on the results of the single markers to decrease multiple testing. Also, we tested for evidence of interactions between markers on GABRB3 and GAD67 by reanalyzing the GABRB3 data stratified by GAD67 alleles. Bonferroni correction was applied for each population group and a p-value lower than 0.0007 (0.05/66) was considered statistically significant for the Iowa population and a p-value lower than 0.001 (0.05/44) was considered statistically significant for the Filipino population.

Table 1 and Table 2 summarize the results for GABRB3. There were no statistically significant results when accounting for the multiple comparisons but under a less strict criterion, the marker rs4477673 showed a trend for association with cases with an affected palate in the Iowa population, and markers rs2059574, rs6576618, and rs981778 showed a trend for association with Filipino cases only presenting a cleft of the lip. However, when we combined Iowa and Filipino data sets for the analysis, the p-values increased, suggesting that the possible associated alleles are not the same in both populations (data not shown). Table 3 summarizes the results for GAD67. No statistically significant results were found, but there was a trend for an association between the marker rs2177433 and clefts involving the lip and the palate in the Iowa population. But as for the analysis with GABRB3, analysis of combined Iowa and Filipino data sets showed increased p-values, suggesting again that the possible associated alleles are not the same in both populations (data not shown). Table 4 presents the results for the only haplotype combination that showed a trend for association. The GABRB3 haplotype rs2059574-rs6576618-rs4477673 showed an association with CL/P in the Philippines. We did not find evidence for interaction between GABRB3 and GAD67 in our data.

Our study provides further evidence of a possible association between GABRB3 and GAD67 with oral clefts. It appears that GABRB3 may contribute differently to the cleft phenotype in Iowans and in Filipinos, with a stronger effect in cases with palate involvement in Iowa, versus an effect in cases with involvement only of the lip in the Philippines. We also found evidence of a possible association between GAD67 and oral clefts in Caucasians. While none of our associations reached formal levels of significance using Bonferroni corrections the p values of 0.05 and lower can at least be hypothesis generating for further studies on larger sample sizes and with improved phenotyping. The inclusion of other clinical characteristics such as presence of dental abnormalities [Letra et al., 2007], or subclinical orbicularis oris muscle defects [Neiswanger et al, 2007] are viable alternatives for subphenotyping CL/P.

In conclusion, we have presented data on the largest sampling to date of oral clefts for GABRB3 and GAD67 markers. Our results are inconclusive but the trend for association we found

suggests further studies are necessary to evaluate the possible contribution of GABRB3 and GAD67 to oral clefts.

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Table 1

GABRB3 association studies in Iowa.

Marker	ALL		CLO		CLP		CPO		CL/P		CLP+CPO	
	N	p-value	N	p-value	N	p-value	N	p-value	N	p-value	N	p-value
rs1426217	76	0.41	16	0.13	21	0.43	21	0.85	37	0.66	42	0.49
rs1593610	88	0.92	16	0.83	33	0.086	15	0.37	49	0.13	48	0.37
rs2059574	90	0.93	18	0.84	28	0.26	19	0.55	46	0.32	47	0.21
rs4477673	89	0.095	18	0.55	30	0.019	19	0.22	48	0.03	49	0.0092
rs4572353	85	0.78	18	0.84	30	0.63	15	0.83	48	0.80	45	0.80
rs6576575	41	0.10	4	0.32	11	0.41	11	0.13	15	0.23	22	0.10
rs6576618	88	0.25	19	0.83	27	0.87	19	0.30	46	1.00	46	0.60
rs890317	78	0.12	11	0.62	25	0.063	19	0.84	36	0.07	44	0.20
rs981778	106	0.73	21	0.083	33	0.35	23	0.47	54	0.72	56	0.81

N = number of informative families; ALL = all clefts; CLO = cleft lip only; CLP = cleft lip with cleft palate; CPO = cleft palate only; CL/P = cleft lip with or without cleft palate.

Table 2

GABRB3 association studies in the Philippines.

Marker	ALL		CLO		p-value		CLP		p-value		CL/P		p-value	
	N		N				N				N			
rs1426217	98		34		0.21	0.41	63		0.34	0.21	97		0.21	0.21
rs1593610	131		32		0.64	1.00	97		0.71	0.75	129		0.75	0.75
rs2059574	171		53		0.13	0.039	114		0.94	0.29	166		0.29	0.29
rs4477673	178		54		0.057	0.91	118		0.16	0.22	172		0.22	0.22
rs4572353	110		27		0.15	0.86	80		0.12	0.20	106		0.20	0.20
rs6576575	112		34		0.50	0.30	77		1.00	0.55	111		0.55	0.55
rs6576618	119		39		0.37	0.029	78		0.84	0.28	117		0.28	0.28
rs890317	149		43		0.30	0.59	103		0.37	0.26	145		0.26	0.26
rs981778	133		38		0.09	0.024	90		0.51	0.07	128		0.07	0.07

N = number of informative families; ALL = all clefts; CLO = cleft lip only; CLP = cleft lip with cleft palate; CL/P = cleft lip with or without cleft palate.

Table 3

GAD67 association studies.

Marker	ALL		CLO		CLP		CPO		CL/P		CLP+CPO	
	N	p-value	N	p-value	N	p-value	N	p-value	N	p-value	N	p-value
Iowa												
rs2177433	84	0.025	10	0.20	23	0.019	24	0.27	34	0.014	48	0.024
rs3749034	68	0.91	13	0.81	19	0.20	14	0.80	33	0.34	34	0.33
Philippines												
rs2059725	60	0.81	21	0.30	41	0.39	-	-	61	0.90	-	-
rs3749034	135	0.20	39	0.37	93	0.25	-	-	131	0.17	-	-

N = number of informative families; ALL = all clefts; CLO = cleft lip only; CLP = cleft lip with cleft palate; CPO = cleft palate only; CL/P = cleft lip with or without cleft palate.

Table 4
GABRB3 haplotype rs2059574-rs6576618-rs4477673 (common allele-rare allele) summary results.

Population	ALL		CLO		CLP		CPO		CL/P		CLP+CPO	
	N	p-value	N	p-value	N	p-value	N	p-value	N	p-value	N	p-value
Iowa	70	0.37	8	0.91	15	0.73	10	0.76	23	0.86	24	0.53
Philippines	130	0.014	40	0.12	67	0.23	-	-	116	0.031	-	-

N = number of informative families; ALL = all clefts; CLO = cleft lip only; CLP = cleft lip with cleft palate; CPO = cleft palate only; CL/P = cleft lip with or without cleft palate.