Brief communication

Lower incidence of nonsyndromic cleft lip with or without cleft palate in females: Is homocysteine a factor?

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In India, as in other parts of the world, nonsyndromic cleft lip with or without cleft palate (NSCL±P) is a highly prevalent birth defect, its incidence in males being twice that in females. A case–control association study has been carried out with respect to homocysteine level and *MTHFR* C677T, A1298C and *SLC19A1* (*RFC1*) G80A genotypes from an eastern Indian cohort to investigate whether Hcy and other Hcy-pathway genes also contribute to the risk level. While *MTHFR* 677T and *SLC19A1* 80G are individually and cumulatively risk factors, *SLC19A1* 80A appears to be protective against *MTHFR* 677T risk allele. Elevated Hcy associates with NSCL±P both in case mothers and cases. Significantly, this difference shows a gender bias: the level of elevation of Hcy in female cases is distinctly higher than in males, and more case females are hyperhomocyteinemic than the case males. It implies that compared with the males, higher level of Hcy is needed for NSCL±P to manifest in the females. We consider this as one of the possible factors why the incidence of this disorder in females is much lower than in males.

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1. Introduction

Elevation in the level of blood homocysteine (Hcy) and polymorphisms/mutations in the Hcy-pathway genes that lead to its elevation have been identified as risk factors for certain congenital (e.g. NTD) and late-age disorders (e.g. cardiovascular, cancers) (Refsum *et al.* 1998; Maron and Loscalzo 2009; Blom and Smulders 2011). This association differs from region to region and between different populations. The frequency of T allele of *MTHFR* C677T, for instance, varies among populations from 1% (Africa, Southeast Asia) to 30% (Europe, America) as well as in association with diseases: lower the frequency higher the chance of it being a risk factor (Rosenberg *et al.* 2002; Gueant-Rodriguez *et al.* 2006; Verkleij-Hagoort *et al.* 2007). Our earlier study on nonsyndromic cleft lip with or without cleft palate (NSCL±P) has shown that the T allele of *MTHFR* C677T is a risk factor both in cases and case mothers (Ali *et al.* 2009). However, in several other populations it is a risk factor either only in cases or case mothers or else in neither of them (Verkleij-Hagoort *et al.* 2007; Blanton *et al.* 2011). This inconsistency against NSCL±P persists with respect to the effect of folic acid, a cofactor for *MTHFR* enzyme action which is protective in some populations (Wilcox *et al.* 2007; Mossey *et al.* 2009) but ineffective in some others (van Rooji *et al.* 2003). However, since at least in some cohorts, *MTHFR* C677T is a risk factor and folic acid supplementation is a preventive, it is likely that elevation of homocysteine level would also be a risk factor for NSCL±P. However, here again the few studies done from different regions in Europe and the US give conflicting results. Barring the report of Wong *et al.* (1999) from the Netherlands, which shows the case mothers to have higher Hcy than the controls, no association is seen in others (Shaw *et al.* 2009; Munger *et al.* 2011). Considering that *MTHFR* SNP 677T is a risk factor in the eastern Indian population, we have measured Hcy levels in a case–control (and case mothers and control mothers) study to evaluate the possibility of Hcy being a risk factor for NSCL±P. In this report we confirm association of elevated Hcy level with

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NSCL±P both in the cases and case mothers. We also show that in the case females the Hcy elevation is significantly higher than in case males, the opposite of what is seen in the controls. We suggest that differential levels of Hcy may partly account for difference in the incidence of NSCL±P in males and females.

2. Materials and methods

2.1 *Sample collection*

While Hcy measurement was performed in 318 cases (males 186; females 132), 281 controls (males 176; females 105), 98 case mothers and 109 control mothers, genotyping was performed on 467 cases and 469 controls which comprised samples in addition to all those in which Hcy was measured. Cases were collected from G. S. Memorial Hospital, Mehmoorganj, Varanasi. Syndromic and familial cases were excluded from the study. Control samples (children as well as mothers) were collected from nearby schools, primary health centres and the university hospital. People having family history of any congenital malformation, kidney-related diseases and other severe diseases were excluded from the study. The control children and mothers, however, were two independent groups with no biological relationship between them. The median age was 5 years (IQR: 1–9 years) for the case children and 9 years (IQR: 3–12.5 years) for the controls. The median age of the mothers, for both the cases and controls, was 26 years (IQR: 24–27 years) and they were matched with respect to parity and passive smoking; none of the mothers was firsthand smoker. Informed consent was taken from each family.

2.2 *Methods*

Plasma Hcy level was measured by reverse phase HPLC using a fluorescence detector following the protocol of Kumar *et al.* (2005). Genotyping of *MTHFR* C677T (rs1801133), A1298C (rs1801131) (Rai *et al.* 2006) and *SLC19A1* [*RFC1*] G80A (rs1051266) (Chango *et al.* 2000) was done on cases and matching controls using PCR-RFLP approach.

Primer sequences (5′-3′) and enzymes used for the PCR-RFLP are listed below:

MTHFR C677T (enzyme used: HinfI): forward/reverse: T G A A G G A G A A G G T G T C T G C G G G A / CCTCACCTGGATGGGAAAGATCC. *MTHFR* A1298C (MboII): forward/reverse CTTTGGGGAGCTGAAG GACTACTAC/CACTTTGTGACCATTCCGGTTT. *SLC19A1* G80A (HhaI): forward/reverse – AGCGT CACCTTCGTCCC/ TCCCGCGTGAAGTTCTTG.

2.3 *Statistical analysis*

Statistical analysis was performed using GraphPad InStat version 3.0. Odds ratio was calculated to assess the risk level and statistical significance was done by chi-square test or Fisher's exact test. Value of $p \leq 0.05$ was considered statistically significant. Comparison between two groups was done by Mann-Whitney U-test.

3. Results

3.1 *Homocysteine level*

In a recent survey of the levels of Hcy, folic acid and vitamin B12 along with the allele frequency in the known polymorphisms of the Hcy-pathway genes of adult population from eastern India, we had shown that the median Hcy level (12.1 μmol/L) in this cohort was much higher than that in European and the American populations (Sukla and Raman 2012). Also, the level of Hcy in males was higher than in females, as reported in most other populations. With this background information, Hcy was measured in the cases, case mothers and appropriate controls. The median Hcy value (14.9 μmol/L vs 12.5 μmol/L; $p<0.001$) and the proportion of individuals with hyper-homocysteinemia (hypHcy; Hcy level≥15 μmol/L) were significantly higher in cases than in the controls [OR (95% CI)=1.99 (1.42–2.77); $p<0.001$] (tables 1, 2). The most remarkable observation, however, was that the median Hcy in the case females was higher than in males (15.4 μmol/L vs 14.2 μmol/L; $p=0.03$; table 3). Considering that among controls, Hcy level was lower in females than males (11.7 μmol/L vs 13.2 μmol/L; $p=0.02$), the observed gender-wise difference in the cases became even more striking: difference between the medians of case females and control females (15.4 μmol/L vs 11.7 μmol/L; $p<0.001$) being much greater than between the case males and control males (14.2 μmol/L vs 13.2 μmol/L; $p=0.05$). Not only the overall level of Hcy was higher in affected females, the number of affected females with hypHcy was greater than males (56% vs 43.5%; table 4). Since Hcy measurement in the case and control children was done years after the delivery of the child, in order to check whether the age factor was responsible for the differences, the Hcy data were stratified in different age segments and compared between the cases and controls and between the genders. Although Hcy level enhanced consistently till about 10 years of age in both the groups (cases and controls), it was always significantly higher in the cases than in controls except the third group i.e. >5–10 years group [age <1 year: 13.1 vs 10.1 ($p=0.03$); 1–5 years: 13.9 vs 12.1 ($p=0.05$); >5–10 years: 16.6 vs 12.8 ($p=0.07$), >10 years: 16.8 vs 13.1 ($p<0.001$); Mann-Whitney U-test] (figure 1).

Table 1. Median of Hcy concentration (μmol/L) in cases and controls

	Cases Median (IQR); (n)	Controls Median (IQR) ; (n)
Total (males+females)	14.9 (11.1–18.7); (n=318)	12.5 (8.7–16.2); (n=281)

$p<0.001$; Mann-Whitney U test

In addition to the cases, 98 case mothers and 109 control mothers were subjected to similar analysis. Even in this group the Hcy level was higher in the case mothers (median 14.3 vs 10.6 μmol/L; $p<0.001$), and the difference was almost similar as between the case and controls (table 5). This group of case mothers bore 39 affected female and 59 affected male children. While the median Hcy level in them was also higher in cases, it did not differ between the mothers of male and female children (14.5 and 13.9 μmol/L).

3.2 *MTHFR and SLC19A1 (RFC1) genotyping*

Association of certain known polymorphisms of Hcy-pathway genes with Hcy level in the general population from this region was recently investigated and *MTHFR* and *SLC19A1* SNPs showed strong association with Hcy level while other SNPs remained neutral (Sukla and Raman 2012). Therefore, *MTHFR* and *SLC19A1* gene SNPs were genotyped in the case and control samples. As expected T allele of *MTHFR* C677T and G of *SLC19A1* G80A showed association with NSCL±P while C of *MTHFR* A1298C was neutral in all the combinations (table 6). The combined genotype of *SLC19A1* G80A and *MTHFR* C677T (GG/TT) showed greater risk than any of them alone [OR (95% CI)= 4.9 (1.2–20.2) $p=0.03$] (table 6). However, the presence of A allele of *SLC19A1* progressively lowered the frequency of cases (against controls) even with the risk allele of *MTHFR* 677T [table 6; see frequency (%) difference of GG/CT and GA/CT between the cases and controls; also, frequency (%) was lower in cases than controls with AA/CT genotype].

Table 2. Frequency of individuals with high and low Hcy levels

	Cases n (%)	Controls n (%)
Individuals with Hcy≥15 μmol/L	155 (48.7)	91 (32.4)
Individuals with Hcy<15 μmol/L	163 (51.3)	190 (67.6)
OR (95% CI); *p* value	1.99 (1.42–2.77); $p<0.001$	

Table 3. Comparison of Hcy level between males and females in cases as well as controls

	Females* Median (IQR); (n)	Males* Median (IQR) ; (n)
Cases ($p=0.03$) #	15.4 (11.5–20.7); (n=132)	14.2 (10.6–17.0); (n=186)
Controls ($p=0.02$) #	11.7 (7.7–16.0) (n=105)	13.2(9.7–16.7); (n=176)
Mann-Whitney U test	$p<0.001$*	$p=0.05$*

and ∗ represent the groups being compared.

4. Discussion

The aim of the present study is to have a collective assessment of the role of Hcy and Hcy-pathway genes as risk factors. Regarding the genotypes, besides our earlier study showing *MTHFR* 677T as a risk factor in this population (Ali *et al.* 2009), a study from China on *SLC19A1* G80A finds 80G as a risk factor with cases of cleft lip only (Vieira *et al.* 2008). The present study confirms the role of *MTHFR* 677T and *SLC19A1* 80G as risk alleles (individually and collectively). In addition, this report shows protective nature of *SLC19A1* 80A even in presence of *MTHFR* 677CT heterozygotes.

Homocyteine and NSCL±P: Hcy level in the cases and case mothers is significantly higher than in the corresponding controls. At least one previous report shows Hcy as a risk factor in case mothers (Wong *et al.* 1999) but investigations on Hcy levels in affected children have not found any significant difference between the cases and controls (van Rooji *et al.* 2003, Shaw *et al.* 2009; Munger *et al.* 2011). NSCL±P being a developmental disorder, gestational environment must naturally be crucial. Therefore, association of high Hcy level in the case mothers is expected. Elevation of Hcy in the case children, however, appears unique to this population. It can be argued that since Hcy has been measured much after birth, and that Hcy level does vary during early childhood, the difference seen between the cases and controls could be because of the age difference between them. That this is not the case has been ascertained by age-wise comparison of Hcy level between the case and

Table 4. Frequency and percentage of cases with high and low Hcy levels

	Female cases n (%)	Male cases n (%)
Individuals with Hcy≥15 μmol/L	74 (56%)	81 (43.5%)
Individuals with Hcy<15 μmol/L	58 (44%)	105 (56.5%)
RR (95% CI); χ^2 (df=1); *p* value	1.34 (1.03–1.75); 4.35; $p=0.04$	

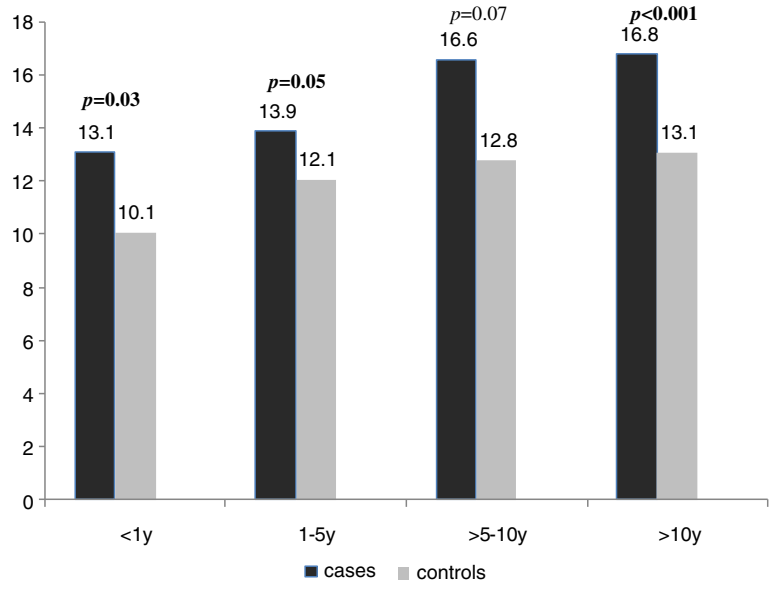

Figure 1. Hcy level in different age groups.

control children and at every stage the level of Hcy in the cases is found to be higher than the controls (figure 1). Interestingly, Munger *et al.* (2011) have made similar observation on folate deficiency in the NSCL±P case mothers, years after the birth of children, in a population from Utah, USA (Munger *et al.* 2011). It implies that the present results, even though retrospectively studied, reflect the condition at birth. An earlier study on the human palatal cell line (HEPM) as well as tissue cultured embryonic lip and palate of mouse has shown that Hcy enters the fetus through amniotic fluid and induces apoptosis of the palatal mesenchyme that prevents fusion of the palate. Obviously, elevated level of Hcy in the fetus is an important risk factor for clefting of palate, and most likely of lip (Knott *et al.* 2003). Walker *et al.* (1999) have shown that Hcy level actually goes down during pregnancy, especially in the first trimester (Walker *et al.* 1999). It is plausible that the trend of raised Hcy level seen in the NSCL±P neonates is an extension of the status in foetus, i.e. Hcy level, though low during pregnancy, would have been higher in the NSCL±P foetus. We also contend that association of Hcy with other developmental disorders (viz, NTD) could have similar physiological etiology.

Table 5. Median of Hcy concentration (μmol/L) in case mothers and control mothers

	Case-mothers (n=98)	Control-mothers (n=109)
Median (IQR)	14.3 (10.8–18.7)	10.6 (8.5–13.4)

$p<0.001$; Mann-Whitney U Test

Table 6. Genotypic distribution of *SLC19A1* and *MTHFR* gene variants in cases and controls

Gene variants	Cases n=467 (%)	Controls n=469 (%)	OR (95% CI)	*p*
		SLC19A1 (*RFC1*) G80A		
GG	181 (38.8)	145 (30.9)	2.2 (1.5–3.3)	<0.001
GA	234 (50.1)	231 (49.3)	1.8 (1.2–2.7)	0.002
AA	52 (11.1)	93 (19.8)	Referent	-
		MTHFR C677T		
CC	327 (70.0)	364 (77.6)	Referent	-
CT	125 (26.8)	100 (21.3)	1.4 (1.03–1.9)	0.04
TT	15 (3.2)	5 (1.1)	3.3 (1.2–9.3)	0.02
		MTHFR A1298C		
AA	191 (40.9)	201 (42.9)	Referent	-
AC	225 (48.2)	223 (47.5)	1.1 (0.8–1.4)	0.7
CC	51 (10.9)	45 (9.6)	1.2 (0.8–1.9)	0.5
AC+CC	276 (59.1)	268 (57.1)	1.1 (0.8–1.4)	0.6
		SLC19A1 G80A/*MTHFR* C677T		
GG/CC	133 (28.5)	115 (24.5)	2.4 (1.5–3.9)	<0.001
GG/CT	41 (8.8)	27 (5.8)	3.2 (1.7–6.0)	<0.001
GG/TT	7 (1.5)	3 (0.6)	4.9 (1.2–20.2)	0.03
GA/CC	158 (33.8)	173 (36.9)	1.9 (1.2–3.03)	0.004
GA/CT	70 (15.0)	57 (12.2)	2.6 (1.5–4.4)	<0.001
GA/TT	6 (1.3)	1 (0.2)	12.7 (1.5–109.2)	0.008
AA/CC	36 (7.7)	76 (16.2)	Referent	-
AA/CT	14 (3.0)	16 (3.4)	1.8 (0.8–4.2)	0.2
AA/TT	2 (0.4)	1 (0.2)	4.2 (0.4–48.1)	0.3

The more intriguing part of the present study, however, is that Hcy level is higher in the case females than in case males. Also, 56% of case females are hyperhomocysteinemic as against 43.5% in case males, even if genotypically *MTHFR* 677CC. This difference is particularly noteworthy because normally Hcy level in females is reported to be lower than in males in adult population (Must *et al.* 2003; Sukla and Raman 2012). Since it is known that difference in Hcy level between male and female children becomes significant mainly during adolescence (Must *et al.* 2003) the data have been analysed in the pre-pubertal age group (<12 year). Assay of Hcy levels within this group (255 cases and 205 controls) reveal no significant gender-wise difference in the controls (males: 13.1; females: 12.3), but in the case children the difference is significant between males (13.9) and females (15.2), implying a genuine difference in the Hcy level between the affected male and female children.

A plausible physiological reason for this difference is not intuitively clear to us. However, it does offer a clue to explain the gender-specific difference in the frequency of NSCL±P, which has a ratio of 2:1 between males and females. NSCL±P is an oft-cited example of the 'threshold' concept of phenotype development in polygenic/multifactorial disorders. We suggest that the threshold level of Hcy required to manifest NSCL±P in females is much higher than in males and that could be one reason why the frequency in females is lower than in males.

Considering that the general population in eastern India suffers from prevalence of hypHcy and vitamin B12 deficiency, and that those having optimum vitamin B12 and folic acid have low Hcy despite genotypic predisposition, it appears that a judicious supplementation of these micronutrients to the prospective mother and during pregnancy could be a preventive against the risk of NSCL±P as well as other developmental disorders in this populous and deprived region of the country.

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