LETTER TO JMG

Cleft lip with or without cleft palate: implication of the heavy chain of non-muscle myosin IIA

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Non-syndromic cleft lip with or without palate (CL/P) is one of the most common malformations among live births, but most of the genetic components and environmental factors involved remain to be identified. Among the different causes, MYH9, the gene encoding for the heavy chain of non-muscle myosin IIA, was considered a potential candidate, because it was found to be abundantly and specifically expressed in epithelial cells of palatal shelves before fusion. After fusion, its expression level was shown to decrease and to become limited to epithelial triangles before disappearing, as fusion is completed.

To determine whether MYH9 plays a role in CL/P aetiology, a family-based association analysis was performed in 218 case/parent triads using single-nucleotide polymorphism (SNP) markers. Pairwise and multilocus haplotype analyses identified linkage disequilibrium between polymorphism alleles at the MYH9 locus and the disease. The strongest deviation from a null hypothesis of random sharing was obtained with two adjacent SNPs, rs3752462 and rs2009930 (global p value = 0.001), indicating that MYH9 might be a predisposing factor for CL/P, although its pathogenetic role needs to be investigated more accurately.

Non-syndromic cleft lip with or without cleft palate (CL/P, MIM 119530) is a common newborn malformation caused by complex and still unknown pathogenetic mechanisms determined by genetic and environmental factors.[1 2] The lip and palatal regions develop from the convergence of different growing processes. Nasal processes and maxillary prominences contribute to the lip, the anterior tooth-bearing alveolus and the anterior palate up to the incisal foramen. As revealed mainly in the mouse, the secondary palate develops as an outgrowth of the maxillary prominences starting from embryo day 11.5. The palatal shelves initially grow vertically down the side of the tongue and then elevate above the tongue as it drops in the oral cavity. With continued horizontal growth, the shelves appose in the midline and fuse by embryo day 15.5. On meeting at the midline of the oropharyngeal cavity, the medial edge epithelia (MEE) of shelves adhere to each other, leading to the medial epithelial seam (MES). The MES initially consists of a multilayered epithelium, and later becomes a single epithelial layer that, through different mechanisms, including epithelial–mesenchymal transformation, apoptosis and cell migration, seals shelves and forms the palate.

Candidate gene studies or genomewide mapping searches for novel loci have led to the identification of genetic factors involved in clefting, such as transcription factors MSX1, IRF6 and TBX22, as well as growth factors transforming growth factor α and transforming growth factor β3 (TGFβ3), or the cell adhesion molecule poliovirus receptor-related 1.[2] Consistent with their role in palatogenesis, they are highly transcribed during palate formation. For instance, TGFβ3 is abundantly and specifically expressed in the MEE cells of pre-fusion shelves, and its expression ceases shortly after the MES is formed, whereas IRF6—responsible for van der Woude syndrome, an autosomal dominant form of cleft lip and palate—is highly expressed in the MEE of the paired palatal shelves immediately before and during their fusion.[3 4]

To improve our understanding of the physiopathological mechanisms responsible for CL/P, we regarded the MYH9 gene as a novel potential candidate because we found that it was highly expressed in palatal shelves.[5] MYH9 encodes for the heavy chain of non-muscle myosin IIA (NMMHCIIA, MIM 160775), one of the three myosins of class II expressed in non-muscle cells to exert contractile force, which is needed for many often unknown cellular functions.[6] We first characterised more deeply its pattern of expression during palatal development in the mouse, and then explored whether the gene was in linkage disequilibrium (LD) with CL/P, using a family-based LD approach.[6] Pairwise and multilocus haplotype analyses identified an LD between MYH9 alleles and the disease, suggesting that determinants of CL/P susceptibility that are not yet identified are localised within the MYH9 locus.

MATERIALS AND METHODS

Myh9 and TGFβ3 in situ hybridisation and immunohistochemistry

A mouse *Myh9* antisense probe was obtained by linearisation of clone AA575507 (IMAGE) with *Sal*I and transcription with SP6 RNA polymerase, while digestion of the same plasmid with *Not*I and transcription with T7 RNA polymerase generated a sense control probe. The mouse *Tgfβ3* probe was obtained by reverse transcription-PCR from the RNA of mouse palate at embryo day 14.5 using the primers Tgfβ3IF (5′-GAGCCCCTGACCATCTTGTAC-3′) and Tgfβ3IR (5′-CCTCTGCTTTTGAGTCCAGC-3′) for amplification. The PCR product was subcloned into the pCRII-TOPO vector (TOPO TA Cloning, Invitrogen, Milan, Italy). The plasmid was linearised either with *BamH*I (antisense probe) or with *Not*I (sense probe), and transcribed with T7 and SP6 RNA polymerase, respectively.

Mouse embryos at day 14.5 were harvested from CD1 pregnant females and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4°C. For in situ hybridisation, sections were analysed as described previously.[5] We used adjacent sections for hybridisation of the two *Myh9* and *Tgfβ3* genes. Slides were coverslipped with 70% glycerol in PBS and photographed (AxioCam digital camera, Zeiss, CarlZeiss, Milan, Italy) using a microscope with Nomarski optics (Axioplan, Zeiss).

Abbreviations: CL/P, cleft lip with or without cleft palate; LD, linkage disequilibrium; MEE, medial edge epithelia; MES, medial epithelial seam; NMMHCIIA, heavy chain of non-muscle myosin IIA; PBS, phosphate-buffered saline; SNP, single-nucleotide polymorphism; TDT, transmission disequilibrium test; TGFβ3, transforming growth factor β3

For immunohistochemical analysis, gelatin tissues were sectioned (15 μm) and mounted on Superfrost-pretreated glass slides (Fisher Scientific, Pittsburgh, Pennsylvania, USA). The mouse sections were fixed in 4% paraformaldehyde in PBS for 15 min and then incubated with a blocking reagent, 10% goat serum in PBS for 1 h. The blocked sections were incubated in the presence and absence of a polyclonal antibody against NMMHCIIA (Covance, Princeton, New Jersey, USA) at dilution 1:200. The sections were then washed with PBS and incubated with a biotin-conjugated antirabbit IgG secondary antibody as described previously.[7]

Sets of families

A sample consisting of 218 patients from Italy with non-syndromic CL/P and their parents was used in this study. Specifically, 65 patients had only cleft lip, whereas 153 had both lip and palate fusion defects. Patients with only cleft palate were excluded from the study. Since it is not clear whether cleft lip and lip and palate fusion defects are separate clinical entities or are different expressions of the same pathology, the two groups were pooled, thus increasing the power of statistical analyses.[8 9] To classify the CL/P as non-syndromic and to exclude potential teratogenic influences, a careful anamnesis was carried out to evaluate the presence of any other somatic or neurological disorders in the family and the use of clefting substances, such as phenytoin, warfarin and ethanol, during pregnancy. Although 131 cases were considered sporadic or non-familial, as no other relatives manifested the malformation, 87 were familiar cases, because of the recurrence of CL/P within each unrelated pedigree. After obtaining informed consent, DNA was extracted from peripheral blood samples as described previously.[10]

Markers

Eight SNPs within the MYH9 locus were analysed for LD (table 1). SNP rs5995288 was selected because it maps in a regulative region, whereas SNP rs2269529 introduces the Ile1626Val protein variant in the NMMHCIIA and might be regarded as an actual susceptibility factor.[11] These two polymorphisms were typed by restriction enzyme digestion of PCR products (primers and protocols are available on request). The other six SNPs were selected from among validated assays, using the Applied Biosystems SNPbrowser Software. Selection was made considering the exon position in the MYH9 locus, and preference was accorded to SNPs with low inter marker LD and minor allele frequency >0.2. Genotypes were obtained using an ABI PRISM 7700 Sequence Detection System and the TaqMan chemistry according to Applied Biosystems (Milan, Italy) protocols.

Statistical analysis

LD between SNP alleles and disease was tested by the transmission disequilibrium test (TDT), which examines the transmission of alleles from heterozygous parents to affected offspring.[12] Pairwise and multilocus haplotype analyses, in a sliding window up to five markers, were performed using the program TDTPHASE, as part of the UNPHASED package.[13] This program provides both global p values, which assess the significance of distortion in transmission for all the test haplotypes, and p values that assess the significance of distortion in transmission for specific haplotypes. The analysis was restricted to phase-certain haplotypes only, in a conditional logistic regression model. This is equivalent to the extended transmission disequilibrium test that ensures a valid test for either genetic linkage or allelic association.[14] A rare haplotype frequency threshold of 0.03 was adopted, because likelihood ratio statistics may be sensitive to rare haplotypes.

LD between markers was also calculated using the D′ and r^2 statistics from parental haplotypes, using the program ldmax of the GOLD package.[15]

RESULTS

MYH9 is highly expressed in the mouse during palate fusion

Our previous studies on *Myh9* in mice during development showed a high expression of the gene in the palate.[5] We looked more accurately into its expression by RNA in situ hybridisation on adjacent sections of the palate at embryo day 14.5, and revealed that the gene was abundantly and specifically expressed in MEE cells of shelves before fusion (fig 1A). Its expression gradually decreases and becomes limited to epithelial triangles after the MES is formed. This pattern of expression overlaps with that of the TGFβ3 gene in both temporal and spatial expression. We also analysed the expression level of the Myh9 product by immunohistochemical analysis. Similar to the mRNA, the protein is expressed at high level, confirming the presence of NMMHCIIA in the MEE cells of paired palate shelves immediately before and during their fusion (fig 1B).

SNPs within MYH9 are in LD with CL/P

Eight MYH9 intragenic SNPs were used to investigate allele and haplotype transmission in a CL/P sample of 218 patient/parent triads. LD between each SNP allele and disease was analysed by standard TDT (table 1).

None of the SNPs tested showed evidence of deviation from the Hardy–Weinberg equilibrium in either affected or unaffected individuals. Significant distortion in allele transmission was detected at the rs3752462 locus, where the nucleotide A allele was found to be transmitted and not transmitted in 116

Table 1 Single-nucleotide polymorphisms selected from within the MYH9 locus and their relative transmission disequilibrium test results

n	dbSNP id	Genome position*	bp to next	Gene position	Alleles†	MAF‡	T/NT§	p Value¶
1	rs5995288	chr22:35087134	16555	Intron 1	G/A	0.36	91/88	0.82
2	rs739097	chr22:35070579	27221	Intron 1	T/C	0.49	85/94	0.50
3	rs2071731	chr22:35043358	4199	Intron 6	C/T	0.41	93/95	0.88
4	rs1002246	chr22:35039159	4476	Intron 10	C/T	0.29	81/81	1.00
5	rs3752462	chr22:35034683	10877	Intron 13	G/A	0.41	84/116	0.02
6	rs2009930	chr22:35023806	14952	Intron 19	T/C	0.40	95/107	0.40
7	rs2269529	chr22:35008854	6440	Exon 33	A/G	0.26	78/83	0.69
8	rs7078	chr22:35002414	—	Exon.40-3′UTR	T/C	0.29	72/84	0.34

MAF, minor allele frequency; SNP, single-nucleotide polymorphism; T/NT, transmission/non-transmission; UTR, untranslated.
*UCSC Genome Browser on Human May 2004 Assembly.
†SNP alleles in coding frame, major allele first.
‡MAF calculated from all parental chromosomes.
§T/NT counts from heterozygous parents are given for the major allele.
¶p value for transmission disequilibrium test.

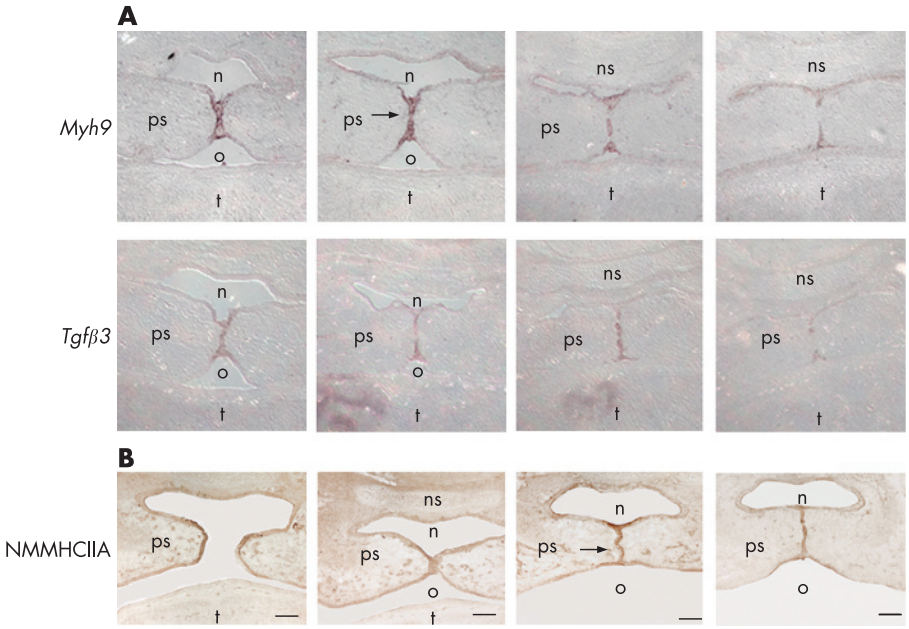

Figure 1 Expression studies during palate fusion in mouse. (A) RNA in situ hybridisation of the *Myh9* and transforming growth factorβ3 (*Tgfβ3*) genes in adjacent sections of embryonic mouse at day 14.5 showing an overlapping pattern of expression. (B) Immunohistochemistry using antibodies against heavy chain of non-muscle myosin IIA (NMMHCIIA) during palate fusion on mouse sections at embryo day 14.5. The arrows indicate the medial epithelial seam (MES) with the highest expression levels of Myh9 mRNA and protein. n, nasal cavity; ns, nasal septum; o, oral cavity; ps, palate shelf; t, tongue. Scale bars represent 100 μm.

and 84 cases, respectively (p = 0.02). Haplotype analysis of multilocus data is potentially a powerful means to detect association in multifactorial diseases.[16] Sliding windows including two to five SNPs were examined. Significant distortion in transmission was observed with different combinations of markers (fig 2). The highest departure from random sharing was observed with two adjacent markers, rs3752462 and rs2009930 (nominal p value <0.001, Bonferroni-adjusted p value <0.001). Combining single-marker and multipoint analyses, we performed 30 association tests, 10 of which produced p values lower than the nominal level of significance. The conservative Bonferroni correction for multiple testing produced an adjusted α level of 0.0017. At this level, we obtained four significant tests in multipoint analysis.

In order to identify any conserved blocks carrying putative CL/P susceptibility mutation(s), we also defined the haplotypes that were significantly overtransmitted to patients with CL/P from heterozygous parents (table 2).

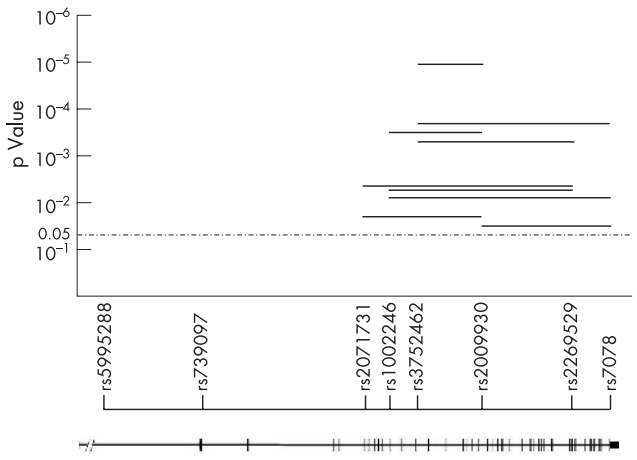

Figure 2 Global p values obtained with multilocus haplotype analyses. p Values are plotted on a log10 scale corresponding to the physical map of the single-nucleotide polymorphisms (SNPs) and exon–intron map of the MYH9 gene.

All the overtransmitted haplotypes are characterised by the presence of either allele A at marker 5 (rs3752462) or allele C at marker 6 (rs2009930), or both A and C. Interestingly, the longer the haplotype, the higher its percentage of transmission. LD between alleles at different loci was examined by means of D′ and r^2 estimation (table 3). A considerable amount of LD—that is, D′>0.9 and r^2>0.45 (typed bold in table 3), was observed between markers 2 and 3, and between markers 5, 6 and 7.

DISCUSSION

Non-syndromic CL/P is a complex trait with no obvious mode of inheritance, and numerous studies have failed to identify genes with any major influence on the disease.[2] The MYH9 gene was analysed in the present study because, as previously observed, it was highly expressed in shelves during palate fusion.[5] Accurate studies revealed that both gene and protein were abundantly expressed in the MEE before fusion; after the MES was formed, their expression remained high but was limited to epithelial triangles and, as fusion was completed, it was no longer detectable. This pattern of expression overlaps with that of the *TGFβ3* gene in both temporal and spatial expression, suggesting that MYH9 might play an important role during palate development. Since we supposed that the molecular mechanisms involved in epithelial fusion and epithelial–mesenchymal differentiation during lip or palate development at least partially overlap, we undertook a family-based association study using a CL/P sample, enrolled previously,[17] to test the hypothesis of MYH9 involvement in CL/P. The analysis of allele and haplotype transmission in 218 patient–parent triads strongly supports an association between CL/P and MYH9. Using both pairwise and multipoint analyses, we performed 30 significance tests to verify the hypothesis. Of note, in our study, 10 out of 30 association tests obtained p values <0.05, when it is accepted that 1 out of 20 is significant by chance alone. Such a result should be indicative of a true association. Methods to correct the significance threshold for multiple testing in genetic association studies with linked markers and in multipoint analysis are still a matter of debate.[18] Bonferroni correction by the number of combinations considered is one method to deal with multiple-testing problems. This method is appropriate for independent tests, but may be judged excessively conservative

Table 2 Single-nucleotide polymorphism alleles and haplotypes overtransmitted to patients with cleft lip with or without cleft palate from heterozygous parents

SNPs											
1	2	3	4	5	6	7	8	T*	NT*	T† (%)	p Value‡
				A				116	84	58	0.014
			C	A				64	38	63	0.014
				A	C			80	48	63	0.015
		T	C	A				36	19	65	0.023
			C	A	C			55	28	66	0.004
					C	G	C	39	20	66	0.017
		T	C	A	C			31	15	67	0.019
				A	C	G	C	39	16	71	0.003
		T	C	A	C	G		25	8	76	0.002
			C	A	C	A	T	16	6	73	0.030
			C	A	C	G	C	20	9	69	0.039

NT, non-transmitted; SNP, single-nucleotide polymorphism; T, transmitted.
*Counts of T and NT haplotypes from heterozygous parents.
†Transmission percentage.
‡ p Value for transmission disequilibrium test.

in the present case. Nevertheless, four tests still proved significant using a crude and conservative Bonferroni-corrected threshold of 0.0017. A maximum value of LD was obtained via multipoint analysis with SNPs rs3752462 and rs2009930 (nominal p value $\leqslant 0.001$, Bonferroni-adjusted p value <0.001), which are localised in the MYH9 genomic region spanning from exon 13 to exon 20.

As in other complex multifactorial diseases, the goal is to identify susceptibility alleles. This is itself a difficult task in complex diseases, and is still more complicated in our case owing to the complexity of MYH9, being a large gene with 40 coding exons spread >100 kb over the genome. Independent LD studies should thus be undertaken to confirm the involvement of MYH9, before proceeding to any fine mutational analysis.

Mutations in the MYH9 gene cause MYH9-related disease (MYH9RD), an autosomal dominant disease characterised by congenital macrothrombocytopenia, NMMHCIIA inclusions in the neutrophils, hearing impairment, cataract and renal failure.[19] At least to the best of our knowledge, no patient with MYH9RD has been reported as having palate defects. However, it is to be noted that there is a very limited spectrum of MYH9 mutations associated with the disease. They consist mainly of missense mutations, occurring to a few amino acids—out of the possible 1961 residues—that are well conserved in evolution but are located in regions of poorly defined functional significance. They also include two in-frame deletions, as well as a few nonsense and frameshift mutations, all localised within a small region at the extreme COOH-terminus (http://www.hgmd.cf.ac.uk/ac/gene.php?gene = MYH9). In particular, four of these residues, 702, 1424, 1881 and 1933, are mutated in >50% of families.

Moreover, whether these mutations determine haploinsufficiency or act through a dominant-negative mechanism is still unclear.[20–23] Specifically, no gross deletions at the MYH9 locus have been identified in patients, which prevents us from confirming haploinsufficiency as the pathogenetic mechanism. In the mouse, by contrast, homozygous destruction of MYH9 is lethal early during embryogenesis, while one knockout allele is not associated with any pathological phenotype, suggesting that the mouse is not a good model for the disease, nor is haploinsufficiency the pathogenetic mechanism of MYH9RD.[24 25] Accordingly, we cannot exclude that MYH9 alleles different from those identified so far might be associated with or predisposed to allelic disorders such as CL/P, although patients with MYH9RD do not manifest palate or lip defects.[26]

Besides the well-characterised role of class II myosins in contraction and force production in muscles, little is known about their specific functional role in non-muscle cells.[6] Some evidence indicates that the non-muscle isoforms have different roles in cytokinesis, phagocytosis, maintenance of shape, organelle/particle trafficking, as well as cell mobility. NMMHCIIA is likely to play a role during the diverse cellular processes implicated in palate fusion, including epithelial–mesenchymal transformation, migration and cell death. Although epithelial–mesenchymal transformation is considered relevant for the degeneration of MEE,[27 28] migration of MEE cells towards the nasal and oral regions has also been proposed as contributing to shelf fusion.[29] Recent data indicate that migration mainly involves periderm cells, ordered migration of which from the basal MEE is a prerequisite for normal fusion.[30] Another mechanism involved in shelf fusion is MEE cell death, which seems to lead to basal lamina degradation and fusion.[31]

Table 3 Linkage disequilibrium (D′ and r^2) between single-nucleotide polymorphisms within the MYH9 locus

SNPs	1	2	3	4	5	6	7	8
1		0.21	0.18	0.04	0.13	0.13	0.19	0.19
2	0.03		**0.92**	0.29	0.61	0.62	0.72	0.21
3	0.03	**0.61**		0.30	0.40	0.40	0.58	0.29
4	0.00	0.04	0.05		0.48	0.46	0.26	0.20
5	0.02	0.26	0.15	0.14		**0.90**	**0.94**	0.27
6	0.01	0.26	0.15	0.13	**0.80**		**0.95**	0.30
7	0.02	0.19	0.17	0.06	**0.46**	**0.48**		0.30
8	0.01	0.02	0.05	0.04	0.04	0.06	0.08	

SNP, single-nucleotide polymorphism.
D′ values are shown above the diagonal, whereas r^2 values are shown below the diagonal; the highest are in bold.

Key points

- We considered MYH9, the gene encoding for the heavy chain of non-muscle myosin IIA, as a potential candidate for non-syndromic cleft lip with or without cleft palate (CL/P) because it was found to be abundantly and specifically expressed in epithelial cells of palatal shelves before fusion.
- Family-based association analysis identified linkage disequilibrium between polymorphism alleles at the MYH9 locus and the disease. The strongest deviation from a null hypothesis of random sharing was obtained with two adjacent single-nucleotide polymorphisms, rs3752462 and rs2009930 (global p value <0.001).
- Expression and genetic data indicate that MYH9 might be a predisposing factor for CL/P, although its pathogenetic role needs to be investigated more accurately.

Turning an epithelial cell into a mesenchymal cell requires alterations in morphology, cellular architecture, adhesion and migration capacity, all mechanisms in which NMMHCIIA is expected to play a fundamental role.[6] Although it is well known that motility is based on actin and myosin, NMMHCIIA function in cell death has not been investigated accurately. Apoptosis leads to morphological changes including cell contraction, dynamic membrane blebbing and nuclear disintegration, the contractile force required for which is generated by actin–myosin cytoskeletal structures.[32]

The absence of the TGFβ3 gene, the high expression level of which overlaps with that of MYH9 during palate fusion, leads to alterations in the development of MEE, with the absence of the filopodia and lamellipodia needed for adhesion and for triggering the fusion event.[33] Consistent with a role of NMMHCIIA in the formation of these structures, it colocalises with the actin bundles and is detected in the rear part of the lamellipodia and in the lamellipodia–cell body transition zone, where it presumably provides force for the cell body to adapt the advancing lamellipodium.[34]

In conclusion, on the basis of the expression data, we conjectured that MYH9 was a candidate gene for CL/P and carried out LD analyses. We found a statistically significant correlation between two SNPs and the disease, suggesting that further analysis of this gene is of fundamental importance for unravelling the pathogenetic mechanisms involved in palate defects.

ELECTRONIC DATABASE INFORMATION

Accession numbers and the URL for data presented herein are as follows:

- Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/
- Human genome draft, http://genome.ucsc.edu/cgi-bin/hgGateway
- GenBank, http://www.ncbi.nlm.nih.gov/Genbank/index.html
- SNP database, http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=snp

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