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Detection of informative markers for searching a causative gene(s) of cleft lip with palate in A/WySn mice

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Abstract Cleft lip with palate is a widespread disfiguring birth defect of complex and poorly understood etiology. The A/WySn mice are good models with which to study the genetic factors of cleft. Our previous study of mated the A/WySn and C3H/He strains found that cleft lip with palate occurred due to cortisone exposure in both the A/WySn and N_2 backcross mice, but not in the C3H/He and F_1 hybrid mice. These findings suggested that autosomal recessive genetic factors cause cleft lip with palate in the A/WySn strain of mice. Interval mapping should identify a candidate chromosome and the region that included the candidate gene(s) causing cleft lip with palate. The present study investigated informative DNA markers for interval mapping. We used 136 Mit (Massachusetts Institute of Technology) markers to amplify target DNA of A/WySn, C3H/He and F_1 hybrid mice by PCR. Amplified DNA products were detected by direct gel analysis. We determined 82 informative markers that were distributed throughout the autosomal chromosome and which could detect polymorphisms between the A/WySn and C3H/He on gel. These results suggest that it is possible to perform the interval mapping for searching candidate chromosome including the loci responsible for cleft lip with palate in A/WySn mice using determined 82 markers.

Key words
A/WySn mice,
C3H/He mice,
Cleft lip with palate,
Mit marker

Introduction

Craniofacial anomalies, particularly cleft lip and cleft palate are major human disfiguring birth defects. Although distinct from isolated cleft palate, cleft lip is usually combined with this condition as cleft lip with palate. The etiology of cleft lip, cleft lip with palate, and cleft palate is complex and might involve major and minor genetic influences with variable interactions from environmental factors[1]. Case-control studies have shown a significant increase in the prevalence of clefts when topical and/or systemic corticosteroids are applied during the first trimester of pregnancy[2,3]. Recently, genetic linkage and association studies have been used extensively to examine candidate genes in clefts. Genetic linkage studies suggest loci for clefts on chromosomes 1[4], 3[5], 4[5,6], 5[5], 6[5], 7[5], 9[5], 10[6], 11[5], 12[5,6], 15[6], 16[5], 20[5] and 21[5]. Linkage disequilibrium and transmission disequilibrium test show association of some genes such as TGFA[7], MSX1[8], TGFB3[8] and human clefts, but other studies have failed to replicate such associations[9,10]. However, which genetic factor(s) causes cleft lip with palate remains inconclusive.

On the other hand, mouse models play an especially important role in disclosing cleft etiologies and in serving as models for environmental co-triggers or interventions. Although generally very rare in laboratory mice, cleft lip with or without palate spontaneously occurs at higher frequencies in the "A" strain derivative, A/WySn mice[11]. Our previous study[12,13] of mated the A/WySn and C3H/He strains found cleft lip with palate in both the A/WySn and N_2 backcross mice such as [A♀ × F_1(A × C3H)♂], [A♀ × F_1(C3H × A)♂], [F_1(A × C3H)♀ × A♂] and

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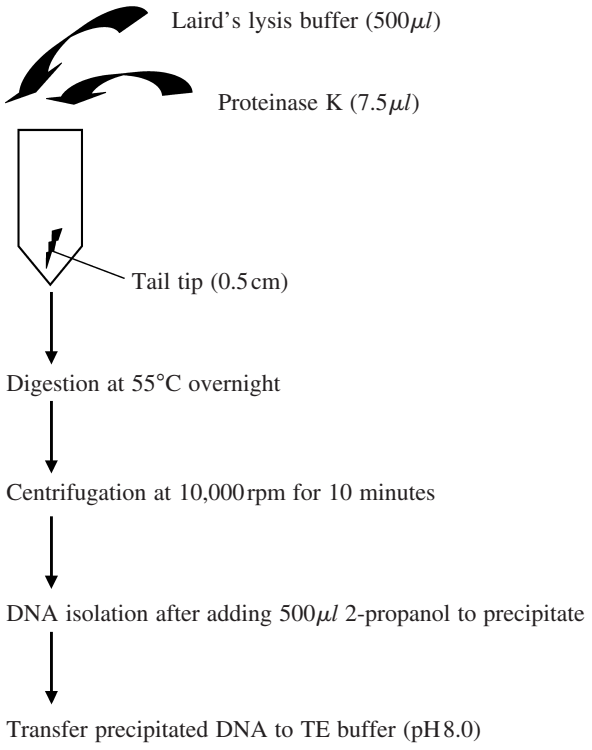

Fig. 1 DNA extraction procedure

[F_1(C3H×A)♀×A♂] (A, A/WySn mice; C3H, C3H/He mice), but not in the C3H/He and F_1 hybrid mice such as (A♀×C3H♂) and (C3H♀×A♂) after cortisone exposure. The ratio between female and male N_2 backcross mice that developed cleft lip with palate were segregated 1:1 according to Mendelian expectation. These findings suggest that cleft lip with palate is caused by autosomal recessive genetic factor(s) in the A/WySn mice. The purpose of this study was to detect polymorphism of DNA products between the A/WySn homozygotes and A/C3H heterozygotes clearly on electrophoresis gel after PCR with use of previously reported informative polymorphic Mit (Massachusetts Institute of Technology) markers that are well distributed throughout the autosomal genome for interval mapping.

Materials and methods

Mice

The A/WySn and C3H/He mice were obtained from the animal facilities at National Institute of Genetics (Shizuoka, Mishima, Japan). The F_1 hybrids were generated from outcross between A/WySn and C3H/He in our laboratory.

I PCR reaction mixtures

Template DNA	1.0μl (total 50 ng)
10×Ex *Taq* buffer	2.0μl
dNTP mixture	1.6μl (200μM)
Ex *Taq* polymerase	0.1μl (0.1 unit)
Forward primer	0.5μl (0.5μM)
Reverse primer	0.5μl (0.5μM)
dH_2O	14.3μl
Total	20.0μl

II PCR conditions

Denaturation	94°C for 3 min	
Denaturation	94°C for 30 sec	35 cycles
Annealing	55°C for 30 sec	
Extension	72°C for 30 sec	
Extension	72°C for 10 min	

Fig. 2 PCR reagents and conditions

DNA

DNA was extracted from the tail tip of A/WySn, C3H/He and F_1 hybrid mice. Figure 1 shows the procedure for DNA extraction as described[14].

Markers

We downloaded whole of Mit markers in existence of autosomal genome from the Mouse Genome Database (http://www.jax.org/). We marked the Mit markers that differed by at least two base pairs in terms of PCR products length between A/J and C3H/He strains. Although A/J and A/WySn strains are distinguished, these two strains always share the same SSLP (simple sequence length polymorphism) alleles, as would be expected by their shared origin as one inbred strain, and in many studies they are treated as equivalent[15]. Then we selected the Mit markers that the individual distance between them were no greater than 35 cM (centi Morgan) from centromeric proximal flanking to distal flanking, based on the effective interval length on the chromosome necessary for detecting linkage by interval mapping was between 25 and 35 cM[16]. These selected markers were purchased from Invitrogen Japan K.K. (Tokyo, Japan).

We amplified DNA from A/WySn, C3H/He, and F_1 mice by PCR using these markers and a programmable PCR thermal cycler 480 (TaKaRa Tokyo, Japan). Figure 2 shows the PCR reagents and

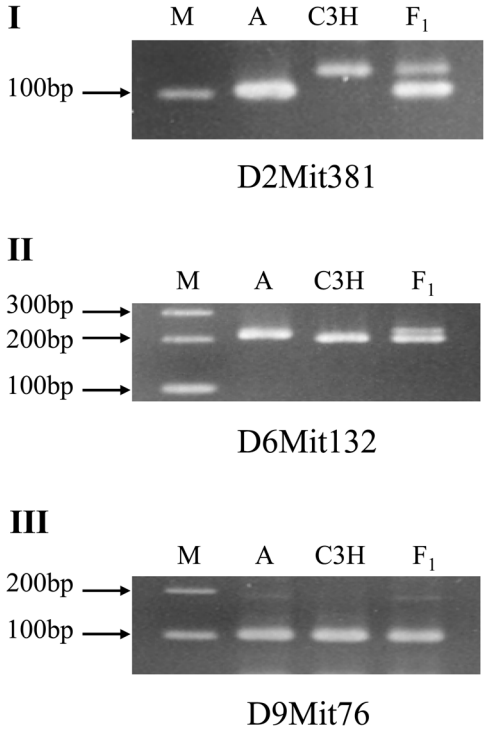

Fig. 3 Examples of amplified markers PCR products to detect polymorphism on gel. Amplified D2Mit381 (I) and D6Mit132 (II) PCR products show detectable polymorphisms between A (104 bp) and C3H (126 bp) and between A (217 bp) and C3H (200 bp), respectively. Amplified D9Mit76 (III) PCR products do not show detectable polymorphisms between A (106 bp) and C3H (112 bp).
M, molecular weight marker (100 bp ladder); A, A/WySn mice; C3H, C3H/He mice; F_1, hybrid from outcross between A and C3H; bp, base pair.

conditions. Amplified DNA products were electrophoretically separated on 4% agarose gel. Then the gels after electrophoresis were visualized by ethidium bromide staining under ultraviolet light to check the recognizable polymorphism between A/WySn and C3H/He, and to distinguish the homozygotes and heterozygotes for A/WySn and F_1.

We reconfirmed the polymorphism of DNA products during the process was achieved as above-mentioned procedures if the amplified products did not show clearly detectable polymorphisms on gel. And when no polymorphisms were seen on gel, the new markers closed those ones were selected.

Results

As shown in Table 1, 136 Mit markers were detected by direct gel electrophoresis. Eighty-two of the Mit markers, for example D2Mit381 and D6Mit132 have 22 and 17 base pair differences between A/WySn and C3H/He, respectively, were able to distinguishable polymorphisms among the A/WySn homozygotes, C3H/He homozygotes and F_1 heterozygotes on gel (Fig. 3I and II). Fifty-four of the Mit markers such as D9Mit76 having more than a two base pair difference between A/WySn and C3H/He, but the amplified PCR products did not show detectable polymorphisms on gel (Fig. 3III). Therefore, we determined 82 informative Mit markers throughout the autosomal genome except a chromosome 9. Informative Mit markers were undetectable on chromosome 9 (Fig. 4).

Discussion

Genetic linkage mapping is useful for finding the chromosomal locations of many recessive and dominant mutant genes[17], and interval mapping is a rapid and efficient way of screening a genome for candidate linkage. For interval mapping, the informative DNA markers require detectable polymorphisms that should distinguish between allele sizes of the parental chromosome, and effective intervals between adjacent markers is necessary. In

Table 1 Cases of detectable polymorphism between A/WySn and C3H/He at 136 Mit markers

Markers	cM	polymorphism	Markers	cM	polymorphism	Markers	cM	polymorphism
D1Mit294	8.3	+	D7Mit247	16.0	+	D12Mit88	25.0	−
D1Mit251	38.1	+	D7Mit91	28.1	+	D12Mit158	38.0	−
D1Mit48	54.0	+	D7Mit181	37.0	+	D12Mit233	52.0	+
D1Mit26	62.1	+	D7Mit31	44.0	−	D12Mit8	58.0	+
D1Mit227	81.6	+	D7Mit126	50.0	+	D13Mit16	10.0	+
D1Mit151	101.0	+	D7Mit186	64.0	+	D13Mit64	30.0	+
D2Mit80	10.0	+	D8Mit289	11.0	−	D13Mit122	36.0	+
D2Mit295	17.0	−	D8Mit224	17.0	+	D13Mit231	39.0	+
D2Mit323	31.7	+	D8Mit65	22.5	+	D13Mit108	45.0	+
D2Mit381	42.6	+	D8Mit231	31.0	−	D13Mit226	59.0	+
D2Mit10	44.0	−	D8Mit304	34.0	−	D13Mit78	75.0	+
D2Mit272	47.5	+	D8Mit242	47.0	+	D14Mit207	5.5	+
D2Mit126	47.5	−	D8Mit211	49.0	−	D14Mit113	25.0	+
D2Mit132	52.5	+	D8Mit167	59.0	−	D14Mit34	40.0	+
D2Mit451	73.2	+	D8Mit91	67.0	+	D14Mit267	60.0	−
D2Mit200	107.0	+	D9Mit361	8.0	−	D15Mit175	9.9	+
D3Mit185	29.5	+	D9Mit88	12.0	−	D15Mit267	10.9	+
D3Mit173	29.5	−	D9Mit66	15.0	−	D15Mit154	18.8	+
D3Mit254	64.1	+	D9Mit224	17.0	−	D15Mit144	32.2	+
D3Mit129	84.9	−	D9Mit326	17.0	−	D15Mit159	49.6	−
D4Mit291	5.0	+	D9Mit191	26.0	−	D16Mit182	3.4	−
D4Mit18	5.2	−	D9Mit93	27.0	−	D16Mit165	10.3	+
D4Mit236	12.1	−	D9Mit172	29.0	−	D16Mit4	27.3	+
D4Mit53	19.8	−	D9Mit106	36.0	−	D16Mit203	55.0	+
D4Mit84	37.6	+	D9Mit304	38.0	−	D16Mit153	56.8	−
D4Mit116	40.0	−	D9Mit340	41.0	−	D17Mit213	9.3	+
D4Mit219	49.6	−	D9Mit8	42.0	−	D17Mit173	11.8	+
D4Mit146	53.6	+	D9Mit238	49.0	−	D17Mit55	12.6	−
D4Mit32	69.8	−	D9Mit76	49.0	−	D17Mit33	18.8	+
D4Mit233	75.5	+	D9Mit121	71.0	−	D17Mit49	23.2	+
D5Mit180	10.0	−	D9Mit151	72.0	−	D17Mit238	34.3	+
D5Mit419	18.0	+	D9Mit322	74.0	−	D17Mit96	54.6	+
D5Mit182	21.0	−	D10Mit3	21.0	+	D18Mit64	2.0	+
D5Mit128	24.0	−	D10Mit115	38.4	+	D18Mit19	2.0	−
D5Mit201	42.0	+	D10Mit230	49.0	+	D18Mit12	17.0	+
D5Mit24	60.0	−	D10Mit180	64.0	+	D18Mit149	24.0	−
D5Mit406	64.0	+	D11Mit71	1.1	+	D18Mit51	37.0	+
D5Mit292	80.0	+	D11Mit306	12.0	+	D18Mit154	47.0	+
D6Mit223	19.0	+	D11Mit135	17.0	−	D18Mit3	54.0	−
D6Mit184	26.4	+	D11Mit5	37.0	−	D19Mit22	5.0	−
D6Mit322	35.2	+	D11Mit298	40.0	+	D19Mit69	6.0	+
D6Mit29	36.5	−	D11Mit321	43.0	−	D19Mit80	22.0	+
D6Mit132	40.0	+	D11Mit145	57.5	+	D19Mit46	24.0	+
D6Mit366	50.5	+	D11Mit10	63.0	+	D19Mit70	51.0	+
D7Mit76	3.4	+	D11Mit104	79.0	+			
D7Mit341	8.0	−	D12Mit58	6.0	+			

+: Represents a detectable polymorphism was detected on gel.
−: Represents a detectable polymorphism was not detected on gel.

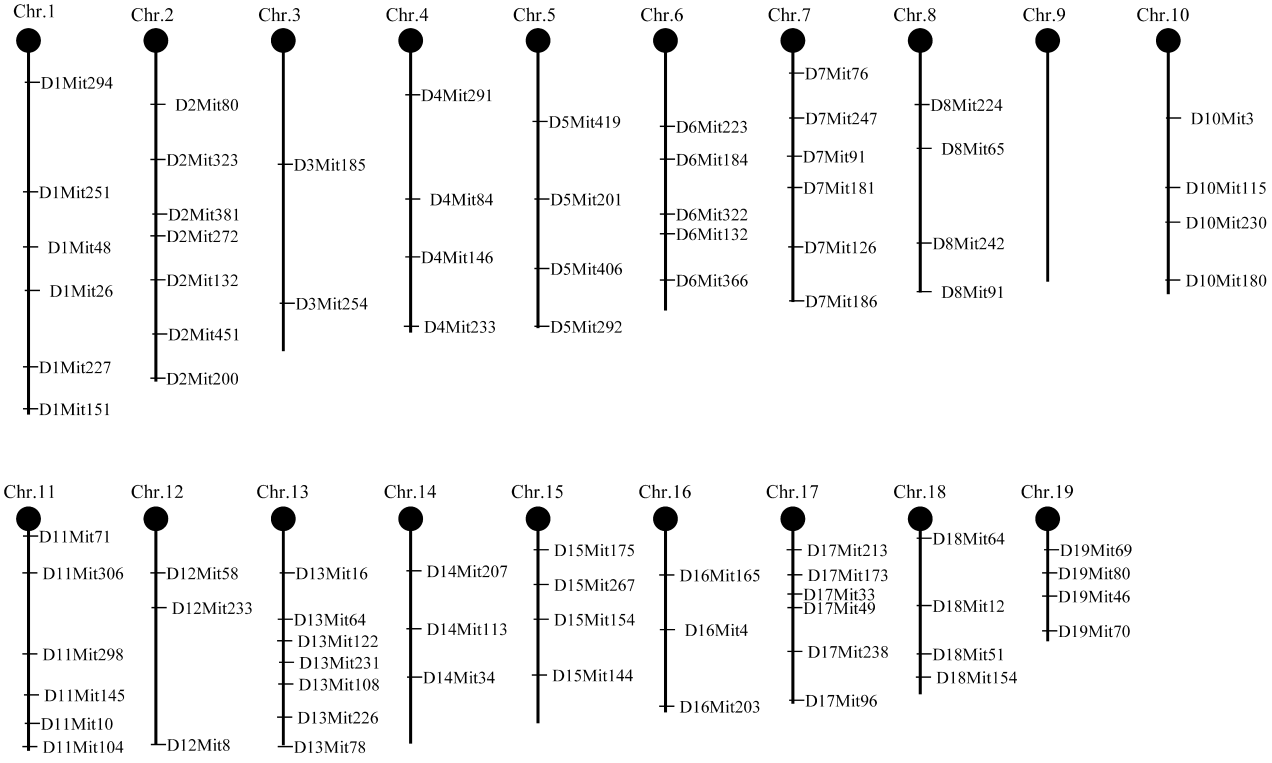

Fig. 4 Chromosomal locations of 82 determined informative Mit markers. The locations of individual loci and lengths of individual chromosomes are taken from the Mouse Genome Database. Centromeric ends are indicated by knobs.

this study, we obtained 82 informative Mit markers that were not only well distributed throughout the autosomal chromosome, but could also detect polymorphisms between A/WySn and C3H/He on gel. These results suggest that interval mapping can be performed to search candidate chromosomes including the cleft lip with palate susceptibility in the A/WySn strain, using the 82 markers determined here and using we have obtained 37 N_2 mice had cleft lip with palate[12] in subsequent experiments.

On the other hand, we noted[12,13] cleft lip with palate occurs, but cleft lip only was not occurs in the A/WySn and N_2 mice during dams pregnancy cortisone exposure. Occurrence of cleft lip with palate by cortisone exposure was thought to be due to a combinative effect between genetic factor of cleft lip and genetic factor of cleft palate, because genetically independent trait, the cause of cleft lip is different from isolated cleft secondary palate[18]. Therefore, using 82 markers, it is suggested that the possibility of searching the loci responsible for cleft lip and cleft palate susceptibilities in the A/WySn strain using subsequent interval mapping.

Recent studies of mouse models relevant to human clefts have discovered some candidate genes/loci, including clf1[18] (between 62.0 to 64.0 cM on chromosome 11), Tcfap2a[19] (at 25.0 cM on chromosome 13), clf2[18] (between 35.0 to 39.0 cM on chromosome 13), Tbx10[20] (at 2.0 cM on chromosome 19) as candidates for cleft lip, and Tgfb2[21] (at 101.5 cM on Chromosome 1), Dlx2[22] (at 44.0 cM on chromosome 2), Msx1[23] (at 21.0 cM on chromosome 5), Gabrb3[24] (at 28.6 cM on chromosome 7), Pax9[25] (at 26.0 cM on chromosome 12), Tgfb3[26] (at 41.0 cM on chromosome 12), H-2 region[27] (in H-2S/H-2D interval on chromosome 17) as candidates for cleft palate. Furthermore, studies of human cleft lip and cleft palate have also found associations between MSX1[8], TGFB2[28], TGFB3[8], GABRB3[29], MTHFR[4] (mouse homologue at 76.4 cM on chromosome 4), TGFA[7] (mouse homologue at 35.8 cM on chromosome 6), BCL3[30] (mouse homologue at 6.5 cM on chromosome 7), RARA[31] (mouse homologue at 57.8 cM on chromosome 11) and human clefts in some families. In this study, we detected D1Mit151, D2Mit381, D5Mit419, D7Mit91, D11Mit10, D11Mit180, D12Mit233, D12Mit8, D13Mit64, D13Mit122, D13Mit231, D17Mit33,

D17Mit49, D19Mit69, D4Mit233, D6Mit322, D7Mit76 and D11Mit145 markers. These markers were not only close to reported candidates such Tgfb2, Dlx2, Msx1, Gabrb3, clf1, Pax9, Tgfb3, Tcfap2a, clf2, H-2S/H-2D region, Tbx10, Mthfr, Tgfa, Bcl3 and Rara, respectively, but also shown detectable polymorphisms between A/WySn and C3H/He on gel. Our results suggest that the association between the reported candidate genes and cleft lip with palate in A/WySn mice induced by cortisone exposure can be detected using these markers.

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