Table 1 Parental ages (year) at childbirth

	Deletion	*TR/GC (M1)*	*TR/GC (M2)*	*MR/PE*	*Epimutation*	*Non-deletion*	*All patients*	*General population*
Maternal age								
Total								
Median	30	37	31	30	38.5	36	32	27.5–30.9
Range	19–42	35–45	29–42	29–37	38–39	30–48	19–48	
Number	84	15	7	3	2	6	117	
Until 2002								
Median	29	37	32	29	—	36	30[a]	
Range	19–42	35–37	29–42	—	—	30–48	19–48	
Number	60	3	5	1	0	6	75	
Since 2003								
Median	32.5	38.5	35.5	33.5	38.5	—	35[a]	
Range	23–39	35–45	30–41	30–37	38–39	—	23–45	
Number	24	15	2	2	2	0	42	
Paternal age								
Total								
Median	32.5	40	35.5	31	41.5	36	33	30.6–33.0
Range	21–47	29–53	28–44	28–37	38–45	33–39	21–53	
Number	82[b]	15	6[c]	3	2	6	114[b,c]	
Until 2002								
Median	32.5	43	35.5	28	—	36	33	
Range	21–47	33–43	28–44	—	—	33–39	21–47	
Number	58[b]	3	4[c]	1	0	6	72[b,c]	
Since 2003								
Median	32.5	39.5	35.5	34	41.5	—	34.5	
Range	22–40	29–53	30–41	31–37	38–45	—	22–53	
Number	24	12	2	2	2	0	42	

Abbreviations: GC, gamete complementation; M1, meiosis 1; M2, meiosis 2; MR,monosomy rescue; PE, post-fertilization mitotic error; TR, trisomy rescue.
The data of the general population indicate the range of the mean parental ages at childbirth from the year 1970 to 2008.
[a]*P*-value=0.00017.
[b]Paternal age was not found in two old patients who had left our follow-up and whose hospital records had been discarded.
[c]Paternal age was not identified in one patient who was born after artificial insemination by donor.

satellite analysis at appropriate institutions. In this regard, considering the opportunity to receive detailed molecular studies, it is possible that upd(15)mat is overlooked more frequently in aged patients than in young patients. If so, this may be relevant to the significant difference in the relative frequency of TR/GC (M1) group between the two time periods ('since the year 2003' versus 'until the year 2002').

In summary, the results imply that the advanced maternal age at childbirth is a predisposing factor for the development of upd(15)mat because of increased M1 errors. This notion is applicable to maternal upd in general, as well as to trisomies. However, there are several caveats as discussed in the above, and the number of patients, especially those classified as TR/GC (M2) group, is small. Thus, further careful studies using a large number of patients are necessary in the future.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Association of Primary Ovarian Insufficiency with a Specific Human Leukocyte Antigen Haplotype (A*24:02-C*03:03-B*35:01) in Japanese Women

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Key Words
Association study · Haplotype analysis · Human leukocyte antigen · Primary ovarian insufficiency · Susceptibility

Abstract
Primary ovarian insufficiency (POI) is a heterogeneous condition defined by the triad of oligo/amenorrhea, elevated gonadotropins and estrogen deficiency in women under the age of 40 years. Although autoimmune abnormalities appear to be involved in the development of POI, there are only a few studies with respect to human leukocyte antigen (HLA). The objective of this study was to identify an HLA allele(s) and/or haplotype(s) constituting a susceptibility factor(s) for POI. We examined 83 Japanese women with apparently idiopathic isolated POI. For controls, Japanese HLA reference data registered in the HLA Laboratory were utilized. No significant association was found for a total of 94 alleles for HLA-A, B, C, DRB1, and DQB1 loci, after both stringent Bonferroni correction and less stringent Benjamini-Hochberg (B-H) correction for multiple comparisons. By contrast, of 86 haplotypes identified for MHC class I (HLA-A, B, and C) and 31 haplotypes detected for MHC class II (HLA-DRB1 and DQB1), a single haplotype (A*24:02-C*03:03-B*35:01) remained significant after Bonferroni and B-H corrections (frequency: 4.82% in women with POI and 1.06% in the control data; p = 0.00049). The results imply that a specific HLA haplotype (A*24:02-C*03:03-B*35:01) constitutes a susceptibility factor for apparently isolated POI in Japanese women.

Primary ovarian insufficiency (POI), or premature ovarian failure, is a heterogeneous condition defined by the triad of oligo/amenorrhea, elevated gonadotropins and estrogen deficiency in women under the age of 40 years [Kalantaridou et al., 1998]. While POI is frequently observed in women with sex chromosome aberrations, it also occurs in women with normal karyotype [Laml et al., 2000]. In this regard, although underlying factors for POI remain to be elucidated in most women with normal karyotype, various genetic and environmental factors have been implicated in the development of POI in such women. Indeed, mutations of several genes including *BMP15* [Di Pasquale et al., 2004], *NOBOX* [Qin et al., 2009] and *NR5A1* (alias *SF-1* and *AD4BP*) [Lourenço et al., 2009] are known to cause POI, as well as premutations

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of *FMR1* [Cronister et al., 1991]. In addition, several candidate genes such as *LHX8* [Qin et al., 2008] and *GDF9* [Kovanci et al., 2007] have been identified to date. Furthermore, endocrine disruptors, chemotherapy, radiation, smoking, and viral infections also constitute risk factors for POI [Morrison et al., 1975; Howell and Shalet, 1998; Sharara et al., 1998; Di Prospero et al., 2004].

Autoimmune abnormalities also appear to be involved in the development of POI in women with normal karyotype. Indeed, POI can occur as a part of autoimmune polyendocrine syndromes [Kauffman and Castracane, 2003]. Moreover, women with POI occasionally have autoimmune hypothyroidism, type 1 diabetes mellitus (T1DM), hypoadrenalism, myasthenia gravis and systemic lupus erythematosus and, conversely, women with autoimmune disorders occasionally exhibit POI [Moncayo-Naveda et al., 1989; Belvisi et al., 1993; Bakalov et al., 2002; Ryan and Jones, 2004]. Non-specific autoantibodies such as antinuclear antibodies are often identified in women with POI [Ishizuka et al., 1999], as are organ-specific autoantibodies such as anti-thyroid autoantibodies [Belvisi et al., 1993] and steroid-producing cell autoantibodies [Betterle et al., 1993].

Human leukocyte antigen (HLA) of the major histocompatibility complex (MHC) plays an essential role in the human immune system. Consistent with this, specific HLA alleles or haplotypes are known to constitute susceptibility factors for several autoimmune disorders such as T1DM [Todd et al., 1988]. For POI, however, there are only a few studies with respect to HLA, and there is no haplotype association study. Thus, we performed HLA allele and haplotype analyses in Japanese women with POI. Here, we focused on apparently idiopathic isolated POI as a relatively homogeneous group, because POI with associated autoimmune abnormalities may represent genetically heterogeneous disorders.

Subjects and Methods

Subjects

This study consisted of 83 Japanese women with apparently idiopathic isolated POI who satisfied the following selection criteria: (1) lack of somatic abnormalities; (2) absence of clinically discernible autoimmune diseases; (3) no history of chemotherapy or radiation; (4) 46,XX karyotype in all the ≥30 lymphocytes examined; (5) no demonstrable mutations in *BMP15, NOBOX, NR5A1, LHX8*, and *GDF9*, and (6) no *FMR1* premutations. Two women were familial cases with a similarly affected sister or mother, and the remaining 81 women were sporadic cases. Organ-specific autoantibodies such as anti-thyroid autoantibodies as well as non-specific antinuclear antibodies were not detected in 60 patients examined, although they were not examined in the remaining 23 patients. The menarchial age ranged from 10 to 15 years (median, 13 years) (menarchial age in normal Japanese girls, 12.25 ± 1.25 years), and the age of POI onset (amenorrhea persisting ≥6 months) ranged from 13 to 39 years (median, 31 years). Serum FSH was above 40 IU/l and estradiol was undetectable or below the normal range on at least 2 occasions. Serum testosterone was undetectable, and polycystic ovary was excluded by abdominal ultrasound studies.

Thus, the following patients with overt autoimmune diseases were excluded from the present study: (1) ten patients with Hashimoto thyroiditis accompanied by anti-thyroid peroxidase autoantibodies; (2) one patient with Basedow disease; (3) one patient with Sjögren syndrome; (4) two patients with systemic lupus erythematosus, and (5) two patients with anti-phospholipid antibody syndrome.

Human Leukocyte Antigen Analysis

This study was approved by the Institutional Review Board Committees of the investigators' affiliations. After obtaining written informed consent, genotyping was performed for HLA-A, B, C, DRB1, and DQB1 loci by the polymerase chain reaction-based sequence-specific oligonucleotide probe method, using leukocyte genomic DNA samples. Haplotype inference was performed for MHC class I (HLA-A, B, and C) and class II (DRB1 and DQB1) by the maximum likelihood method using expectation maximization algorithm [Excoffier and Slatkin, 1995] implemented in the software LDSUPPORT [Kitamura et al., 2002]. For controls, we utilized Japanese HLA reference data registered in the HLA Laboratory (http://www.hla.or.jp/) that have been obtained from 14,631 control subjects for HLA-A, B, and DRB1 loci, 8,240 control subjects for HLA-C locus and MHC class I haplotype, and 2,934 control subjects for HLA-DQB1 locus and MHC class II haplotype. The sex ratio was nearly equal in the control subjects, and HLA allele and haplotype patterns have been shown to be comparable between men and women control subjects (http://www.hla.or.jp/).

Statistical Analysis

Allele and haplotype frequencies were compared between women with POI and control subjects by the Fisher's exact probability test, using R environment version 2.7.1 (http://www.r-project.org/). The odds ratio and the 95% confidence interval were also calculated using the same environment. To address the problem of multiple comparisons, the Bonferroni correction and the Benjamini-Hochberg (B-H) correction were employed in this study [Shaffer, 1995]. The Bonferroni correction is the most stringent method that sets the corrected significance level by dividing the empirical significance level (0.05) by the number of tests, and corrected p values by multiplying the observed p values by the number of tests. The B-H correction is a subsequently developed less stringent method that defines the significance level by multiplying the Bonferroni significance level by the frequency order of observed p values, and corrected p values by dividing the Bonferroni corrected p values by the frequency order of observed p values. In both corrections, observed p values are regarded as significant when corrected p values remain below the empirical significance level (0.05). Since individual HLA genotyping data are not available in the control reference data, it was impossible to perform the permutation test that is frequent-

Table 1. Representative results of the association studies between POI and each HLA allele ($p < 0.05$)

Allele	Frequency, %		Statistical analysis			Bonferroni correction		Benjamini-Hochberg correction		
	women with POI	control subjects	p value	odds ratio	95% CI	significance level	corrected p	significance level	corrected p	frequency order of p value
B*27:07	0.60	0.00	0.0056	530.4	21.51–13,080	0.0017	0.16	0.0017	0.16	1
B*35:03	0.60	0.00	0.0056	530.4	21.51–13,080	0.0017	0.16	0.0017	0.16	1
B*51:02	1.20	0.19	0.034	6.60	1.59–27.29	0.0017	1.00	0.0069	0.25	4
B*55:04	1.20	0.13	0.0071	9.38	2.24–39.21	0.0017	0.21	0.0052	0.07	3
C*15:05	0.60	0.02	0.043	24.96	2.77–224.70	0.0033	0.65	0.0033	0.64	1
DRB1*04:01	3.01	0.94	0.019	3.29	1.34–8.07	0.0023	0.42	0.0023	0.41	1
DRB1*12:02	4.22	1.82	0.045	2.38	1.11–5.09	0.0023	0.99	0.0068	0.33	3
DRB1*14:01	6.63	3.36	0.035	2.04	1.11–3.78	0.0023	0.77	0.0046	0.38	2

CI = Confidence interval.

ly utilized to estimate significant p values in multiple comparisons [Shaffer, 1995].

The power ($1 - \beta$ [type II error]: the probability of rejecting a false null hypothesis) was estimated using GDesignPlus (StaGen Co., Ltd.). The power of >50% is usually required to approve the results obtained in a pilot study like the present study, while the power of >80% is usually necessary to admit the results obtained in a replication study [Cohen, 1988]. Furthermore, false positive report probability (FPRP), the probability of no true association between a genetic variant and disease given a statistically significant finding, was also calculated using α (the significance level), power ($1 - \beta$), and π (assumed frequency of susceptibility allele or haplotype; in this study, π was assumed as 0.02) [Wacholder et al., 2004]. The FPRP of <5% is usually regarded as verifying the quality of the obtained data.

Results

HLA genotyping identified 16 alleles for the HLA-A locus, 29 alleles for HLA-B, 15 alleles for HLA-C, 22 alleles for HLA-DRB1, and 12 alleles for HLA-DQB1 in women with POI (online suppl. table 1; for all suppl. material, see www.karger.com/doi/10.1159/000330122). While low p values ($p < 0.05$) were identified for 8 of these alleles (table 1), none of them were significant after the Bonferroni and B-H corrections.

Haplotype estimation indicated 86 haplotypes for MHC class I and 31 haplotypes for MHC class II in women with POI (online suppl. table 2). Low p values ($p < 0.05$) were identified for 34 of these haplotypes (table 2), and one of them (A*24:02-C*03:03-B*35:01) remained significant after the Bonferroni and B-H corrections. This haplotype was invariably present in a heterozygous condition, and accounted for 4.82% of haplotypes in women with POI (the 2nd most frequent haplotype in this group) and 1.06% of haplotypes in the control subjects (the 15th most common haplotype in this group). The power was calculated as 72.2%, and the FPRP was 3.8%. Notably, each of the A*24:02, C*03:03, and B*35:01 alleles constituting the specific haplotype was fairly common in both women with POI and control subjects and was identified with similar frequencies (online suppl. table 1). There was no haplotype that was regarded as significant by the B-H correction but not by the Bonferroni correction.

Discussion

We studied Japanese patients with apparently idiopathic isolated POI who had no associated overt autoimmune diseases. This selection was performed to reduce genetic heterogeneity among patients. Indeed, POI in patients with Sjögren syndrome and systemic lupus erythematosus may represent specific groups of POI, as well as POI in patients with single gene diseases such as autoimmune polyendocrine syndromes [Kauffman and Castracane, 2003]. Similarly, thyroid disease-associated POI may also exhibit a specific group of POI. Thus, we excluded such patients from this study. It should be pointed out, however, that not all patients with POI received examination of autoantibodies, and that several patients may have subclinical autoimmune diseases or may develop autoimmune diseases at later ages.

The present study revealed significant association between apparently isolated POI and a specific HLA haplotype (A*24:02-C*03:03-B*35:01) in Japanese women. In this regard, since the frequency of each allele for the specific haplotype was similar between women with POI and

Table 2. Representative results of the association studies between POI and each HLA haplotype ($p < 0.05$)

Haplotype	Frequency, %		Statistical analysis			Bonferroni correction		Benjamini-Hochberg correction		
	women with POI	control subjects	p value	odds ratio	95% CI	significance level	corrected p	significance level	corrected p	frequency order of p value
A*02:01-C*01:02-B*54:01	2.41	0.73	0.036	3.37	1.23–9.23	0.00058	1.00	0.01	0.13	23
A*02:01-C*03:03-B*55:04	1.20	0.07	0.0083	16.74	3.72–75.40	0.00058	0.71	0.0017	0.24	3
A*02:01-C*03:04-B*40:02	2.41	0.66	0.026	3.74	1.36–10.28	0.00058	1.00	0.01	0.13	17
A*02:01-C*08:01-B*40:02	0.60	0.00	0.01	298.70	12.12–7,366	0.00058	0.86	0.0023	0.22	4
A*02:01-C*08:01-B*48:01	1.81	0.16	0.0029	11.65	3.49–38.88	0.00058	0.25	0.0012	0.12	2
A*02:06-C*01:02-B*15:01	0.60	0.00	0.01	298.70	12.12–7,366	0.00058	0.86	0.0023	0.22	4
A*02:06-C*01:02-B*40:06	0.60	0.01	0.03	49.93	4.50–553.7	0.00058	1.00	0.01	0.14	18
A*02:06-C*07:02-B*67:01	0.60	0.02	0.049	24.96	2.77–224.7	0.00058	1.00	0.02	0.16	27
A*02:07-C*01:02-B*54:01	0.60	0.02	0.049	24.96	2.77–224.7	0.00058	1.00	0.02	0.16	27
A*02:10-C*01:02-B*15:01	0.60	0.01	0.03	49.93	4.50–553.7	0.00058	1.00	0.01	0.14	18
A*11:01-C*03:04-B*15:01	0.60	0.00	0.01	298.70	12.12–7,366	0.00058	0.86	0.0023	0.22	4
A*11:01-C*03:04-B*51:01	0.60	0.00	0.01	298.70	12.12–7,366	0.00058	0.86	0.0023	0.22	4
A*11:01-C*07:02-B*40:02	0.60	0.00	0.01	298.70	12.12–7,366	0.00058	0.86	0.0023	0.22	4
A*11:01-C*08:03-B*15:01	0.60	0.00	0.01	298.70	12.12–7,366	0.00058	0.86	0.0023	0.22	4
A*24:02-C*01:02-B*46:01	2.41	0.80	0.047	3.06	1.12–8.37	0.00058	1.00	0.02	0.16	26
A*24:02-C*01:02-B*56:03	0.60	0.01	0.03	49.93	4.50–553.7	0.00058	1.00	0.01	0.14	18
A*24:02-C*03:03-B*35:01	4.82	1.06	0.00049	4.75	2.30–9.81	0.00058	0.042	0.00058	0.042	1
A*24:02-C*04:01-B*27:07	0.60	0.00	0.01	298.70	12.12–7,366	0.00058	0.86	0.0023	0.22	4
A*24:02-C*08:01-B*07:05	0.60	0.00	0.01	298.70	12.12–7,366	0.00058	0.86	0.0023	0.22	4
A*24:02-C*08:01-B*40:01	0.60	0.02	0.049	24.96	2.77–224.7	0.00058	1.00	0.02	0.16	27
A*26:01-C*01:02-B*15:01	0.60	0.02	0.049	24.96	2.77–224.7	0.00058	1.00	0.02	0.16	27
A*26:01-C*03:04-B*40:01	1.20	0.18	0.049	6.69	1.58–28.22	0.00058	1.00	0.01	0.17	25
A*26:01-C*07:02-B*39:02	0.60	0.01	0.03	49.93	4.50–553.7	0.00058	1.00	0.01	0.14	18
A*26:01-C*08:01-B*35:01	1.20	0.17	0.036	7.17	1.69–30.34	0.00058	1.00	0.01	0.13	24
A*26:01-C*12:02-B*52:01	1.20	0.21	0.0499	5.90	1.41–24.77	0.00058	1.00	0.02	0.14	31
A*26:01-C*14:02-B*51:01	1.81	0.26	0.01	7.20	2.21–23.48	0.00058	0.86	0.0093	5.5E-02	16
A*29:01-C*15:05-B*15:02	0.60	0.00	0.01	298.70	12.12–7,366	0.00058	0.86	0.0023	0.22	4
A*31:01-C*03:04-B*51:02	0.60	0.00	0.01	298.70	12.12–7,366	0.00058	0.86	0.0023	0.22	4
A*31:01-C*07:02-B*67:01	0.60	0.01	0.03	49.93	4.50–553.7	0.00058	1.00	0.01	0.14	18
A*32:01-C*15:02-B*35:03	0.60	0.00	0.01	298.70	12.12–7,366	0.00058	0.86	0.0023	0.22	4
A*33:03-C*07:02-B*67:01	0.60	0.00	0.01	298.70	12.12–7,366	0.00058	0.86	0.0023	0.22	4
DRB1*04:01-DQB1*03:01	3.01	1.06	0.046	2.91	1.15–7.33	0.0016	1.00	0.0048	0.48	3
DRB1*12:02-DQB1*03:01	4.22	1.70	0.034	2.54	1.16–5.55	0.0016	1.00	0.0032	0.53	2
DRB1*14:06-DQB1*06:02	0.60	0.00	0.0039	106.40	4.31–2,623	0.0016	0.12	0.0016	0.12	1

CI = Confidence interval.

the control subjects, this would imply that an additive or a synergic effect of the alleles constituting the specific haplotype increases susceptibility to the development of POI. Thus, this study exemplifies the importance of haplotype analysis in the investigation of genetic susceptibility.

The mechanism(s) by which this specific haplotype raises the susceptibility to POI remains to be clarified. It may be possible, however, that this haplotype has some ovary-specific effect such as the production of hitherto unknown autoantibodies against an ovary-specific tissue(s), because this haplotype was identified as a susceptibility factor for the apparently isolated POI, and because there is no study suggesting a positive association between this haplotype and non-ovarian autoimmune disorders. In support of this notion, it is known in T1DM that pancreatic β-cell-specific insulinoma-associated antigen 2 autoantibodies are preferentially detected in patients with a specific HLA allele [Qu and Polychronakos, 2009].

HLA association studies have previously been performed for POI in non-Japanese populations. Walfish et al. [1983] found that the prevalence of DR3 antigen was significantly higher in 19 women with POI than in 80 control women. Anasti et al. [1994] reported that the frequency of DR4 antigen was significantly higher in 102

women with POI than in the reference data obtained from 1,927 subjects, whereas it was similar between the 102 women with POI and 102 control women. Jaroudi et al. [1994] identified no significant difference in the frequency of HLA loci between 37 women with POI and 100 control women. In these studies, however, HLA typing was performed by classic serological methods rather than refined genotyping methods, and haplotype analysis was not performed, while the Bonferroni correction was utilized to cope with multiple comparisons. Thus, while a possible association of POI with DR3 and/or DR4 may be suggested, this matter awaits further investigations. In this context, it may be worth pointing out that the present study failed to identify a positive association between POI and DR3 (DRB1*03) or DR4 (DRB1*04) (online suppl. table 1), although this may primarily be due to the ethnic difference, as has been shown in T1DM [Thomson et al., 2007].

Several points should be made with respect to the present study. First, the specific HLA haplotype accounts for only a minor portion (4.82%) of haplotypes identified in women with POI. Second, affected women may have some unidentified pathologic cause(s) for POI, such as mutations of hitherto unknown causative genes. Third, there may be some hidden polygenic and environmental differences between women with POI and the control subjects. Finally, it remains to be determined whether similar results can be reproduced in other studies. These matters imply that the etiologies of POI still remain elusive and await further investigations in most women with POI.

Despite the above caveats, the present study provides a useful clue to clarify the underlying factors for the development of POI. In summary, we propose that a specific HLA haplotype (A*24:02-C*03:03-B*35:01) constitutes a susceptibility factor for apparently isolated POI in Japanese women.

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Aromatase Excess Syndrome: Identification of Cryptic Duplications and Deletions Leading to Gain of Function of *CYP19A1* and Assessment of Phenotypic Determinants

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Context: Aromatase excess syndrome (AEXS) is a rare autosomal dominant disorder characterized by gynecomastia. Although cryptic inversions leading to abnormal fusions between *CYP19A1* encoding aromatase and its neighboring genes have been identified in a few patients, the molecular basis remains largely unknown.

Objective: The objective of the study was to examine the genetic causes and phenotypic determinants in AEXS.

Patients: Eighteen affected males from six families participated in the study.

Results: We identified three types of heterozygous genomic rearrangements, *i.e.* a 79,156-bp tandem duplication involving seven of 11 noncoding *CYP19A1* exons 1, a 211,631-bp deletion involving exons 2–43 of *DMXL2* and exons 5–10 of *GLDN*, and a 165,901-bp deletion involving exons 2–43 of *DMXL2*. The duplicated exon 1 functioned as transcription start sites, and the two types of deletions produced the same chimeric mRNA consisting of *DMXL2* exon 1 and *CYP19A1* coding exons. The *DMXL2* exon 1 harbored a translation start codon, and the *DMXL2/CYP19A1* chimeric mRNA was identified in only 2–5% of *CYP19A1*-positive transcripts. This was in contrast to the inversion-mediated chimeric mRNA that had no coding sequence on the fused exon 1 and accounted for greater than 80% of *CYP19A1*-positive transcripts. *CYP19A1* was expressed in a limited number of tissues, whereas its neighboring genes involved in the chimeric mRNA formation were expressed widely.

Conclusions: This study provides novel mechanisms leading to gain of function of *CYP19A1*. Furthermore, it appears that clinical severity of AEXS is primarily determined by the tissue expression pattern of relevant genes and by the structural property of promoter-associated exons of chimeric mRNA. (***J Clin Endocrinol Metab* 96: E1035–E1043, 2011**)

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Abbreviations: AEXS, Aromatase excess syndrome; CGH, comparative genomic hybridization; E_2, estradiol; FISH, fluorescence *in situ* hybridization; hCG, human chorionic gonadotropin; LCL, lymphoblastoid cell line; NMD, nonsense-mediated mRNA decay; RACE, rapid amplification of cDNA ends; SF, skin fibroblast; T, testosterone.

Aromatase is a cytochrome P450 enzyme that plays a crucial role in the estrogen biosynthesis (1). It catalyzes the conversion of Δ4-androstendione into estrone and that of testosterone (T) into estradiol (E_2) in the placenta and ovary as well as in other tissues such as the fat, skin, bone, and brain (1). It is encoded by *CYP19A1* consisting of at least 11 noncoding exons 1 and nine coding exons 2–10 (Supplemental Fig. 1, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org) (2, 3). Each exon 1 is accompanied by a tissue-specific promoter and is spliced alternatively onto a common splice acceptor site at exon 2, although some transcripts are known to contain two of the exons 1, probably due to a splice error (2, 4). Of the 11 exons 1, exon I.4 appears to play a critical role in the regulation of estrogen biosynthesis in males because this exon contains a major promoter for extragonadal tissues including the skin and fat (2).

Excessive *CYP19A1* expression causes a rare autosomal dominant disorder known as aromatase excess syndrome (AEXS) (5–8). AEXS is characterized by pre- or peripubertal onset gynecomastia, advanced bone age from childhood to the pubertal period, and short adult height in affected males (5–8). Affected females may show several clinical features such as macromastia, precocious puberty, irregular menses, and short adult height (6–8). In this regard, previous studies have identified four heterozygous cryptic inversions around *CYP19A1* in patients with AEXS (5, 8). Each inversion results in the formation of a chimeric gene consisting of a noncoding exon(s) of a neighboring gene (*CGNL1*, *MAPK6*, *TMOD3*, or *TLN2*) and coding exons of *CYP19A1*. Because this condition is predicted to cause aberrant *CYP19A1* expression in tissues in which each neighboring gene is expressed, such inversions have been regarded to be responsible for AEXS (5, 8).

However, such inversions have been revealed only in a few patients with AEXS, and, despite extensive studies, no other underlying genetic mechanisms have been identified to date (6, 8–10). Here we report novel genomic rearrangements in AEXS and discuss primary phenotypic determining factors in AEXS.

Patients and Methods

Patients

This study was approved by the Institutional Review Board Committee at the National Center for Child Health and Development and was performed after obtaining informed consent. We examined 18 male patients aged 8–69 yr (cases 1–18) from six unrelated families A-F (Fig. 1A). The probands were ascertained by bilateral gynecomastia (Fig. 1B) and the remaining 12 males by familial studies. Ten other males allegedly had gynecomastia. There were four obligatory carrier females.

Phenotypic assessment showed pre- or peripubertal onset gynecomastia in all cases, small testes and fairly preserved masculinization in most cases, obvious or relative tall stature in childhood and grossly normal or relative short stature in adulthood, and age-appropriate or mildly advanced bone ages (Table 1) (for detailed actual data, see Supplemental Table 1). Such clinical features, especially gynecomastia, tended to be milder in cases 1–4 from families A and B than in the remaining cases from families C-F. Fertility or spermatogenesis was preserved in all adult cases (≥20 yr). In addition, the obligatory carrier females from families B and D had apparently normal phenotype, and such females from families E and F exhibited early menarche (9.0 yr) and short adult stature (−2.8 SD), respectively.

Blood endocrine studies revealed that LH values were grossly normal at the baseline and variably responded to GnRH stimulation, whereas FSH values were low at the baseline and responded poorly to GnRH stimulation, even after preceding GnRH priming (Table 1) (for detailed actual data, see Supplemental Table 1) (see also Fig. 1C for the cases aged ≥15 yr). Δ^4-Androstendione, T, and dihydrotestosterone values were low or normal. A human chorionic gonadotropin (hCG) test indicated relatively low but normal T responses in five young cases. In most cases, estrone values were elevated, E_2 values were normal or elevated, and E_2/T ratios were elevated. These endocrine data were grossly similar among cases 1–18.

Aromatase inhibitor (anastrozole, 1 mg/d) was effective in all the four cases treated (Supplemental Table 1) (see also Fig. 1C for cases aged ≥15 yr). Gynecomastia was mitigated within 6 months of treatment, and endocrine data were ameliorated within 1 month of treatment.

Primers

Primers used in this study are shown in Supplemental Table 2.

CYP19A1 mRNA levels and aromatase activities

We analyzed relative mRNA levels of *CYP19A1* and catalytic activities of aromatase in skin fibroblasts (SF) and lymphoblastoid cell lines (LCL). mRNA were extracted by a standard method and were subjected to RT-PCR using a high capacity RNA-to-cDNA kit (Life Technologies, Carlsbad, CA). A relative amount of *CYP19A1* mRNA against *B2M* was determined by the real-time PCR method using the Taqman gene expression assay on ABI PRISM 7500fast (Life Technologies) (assay no. Hs00903411_m1 for *CYP19A1* and Hs99999907_m1 for *B2M*). PCR was performed in triplicate. Aromatase activity was determined by a tritium incorporation assay (11). In brief, the samples were incubated with androstenedione-2-^{3}H for 2 h, and $^3H\,H_2O$ in the supernatant of the culture media was measured with a scintillation counter LSC-5100 (Aloka, Tokyo, Japan).

Sequence analysis of *CYP19A1*

Leukocyte or SF genomic DNA samples from the six probands and additional four male patients (Fig. 1A) were PCR amplified for the coding exons 2–10 and their flanking splice sites of *CYP19A1*. Subsequently the PCR products were subjected to direct sequencing from both directions on CEQ 8000 autosequencer (Beckman Coulter, Fullerton, CA).

Genome structure analysis

Oligonucleotide array-based comparative genomic hybridization (CGH) analyses were carried out using a custom-built

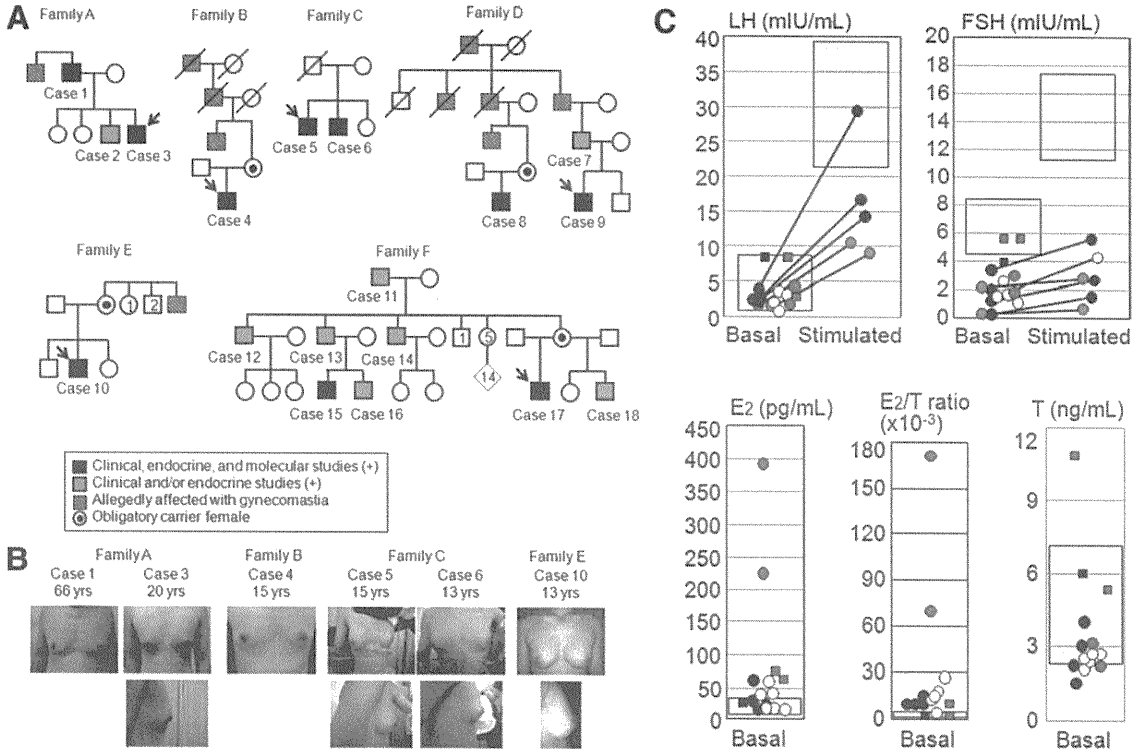

FIG. 1. Summary of clinical data. A, Pedigrees of six families with patients exhibiting AEXS-compatible phenotype. Families A–E are of Japanese origin, and family F is of German origin. Cases from families A–D were hitherto unreported, whereas those from families E and F have previously been described as having AEXS phenotypes (6, 8). B, Gynecomastia of six cases. C, Endocrine data in cases 15 yr of age or older. The *black*, *white*, and *red colors* represent the data in cases of the duplication, the deletion, and the inversion types, respectively; the *blue color* indicates the data of GnRH test after GnRH priming in two cases of the duplication type. The data at the time of diagnosis are denoted by *circles*, and those on aromatase inhibitor (anastrozole) treatment (1 mg/d in the duplication and the deletion types and 2–4 mg/d in the inversion types) are depicted by *squares*. The *light purple areas* represent the normal reference ranges.

oligo-microarray containing 90,000 probes for the 15q11.2-q26.3 region and approximately 10,000 reference probes for other chromosomal region (2 × 105K format, design identification 026533) (Agilent Technologies, Palo Alto, CA). The procedure was as described in the manufacturer's instructions. Fluorescence *in situ* hybridization (FISH) analysis was performed for lymphocyte or SF metaphase spreads, using long PCR products (FISH probes 1 and 2) for rearranged regions and CEP 15 probe for *D15Z4* used as an internal control (Abbott, Abbott Park, IL). The FISH probes 1 and 2 were labeled with digoxigenin and detected by rhodamine antidigoxigenin, and the CEP 15 probe was detected according to the manufacturer's protocol.

Characterization of the duplications and deletions

The duplication junctions were determined by direct sequencing for standard PCR products obtained with a variety of combinations of primers hybridizing to different positions within the *CYP19A1* exons 1 region. The deletion junctions were identified by direct sequencing of the long PCR products obtained with primer pairs flanking the deletions. The sizes of duplications and the deletions were determined by comparing obtained sequences with NT_010194 sequences at the National Center for Biotechnology Information Database (http://www.ncbi.nlm.nih.gov/; Bethesda, MD). The presence or absence of repeat sequences around the breakpoints was examined with Repeatmasker (http://www.repeatmasker.org).

For mRNA analysis, we preformed 5′-rapid amplification of cDNA ends (RACE) using a SMARTER RACE cDNA amplification kit (Takara Bio, Ohtsu, Japan). For both duplications and deletions, first PCR was carried out using the forward primer mix provided in the kit (Universal primer A mix) and an antisense reverse primer specific to *CYP19A1* exon 3 (RACE Rev). Second PCR was carried out for diluted products of the first PCR, using the nested forward primer of the kit (Nested universal primer A) and a reverse primer for *CYP19A1* exon 2 (Nested Rev). For duplications, furthermore, second PCR was also performed using various combinations of primers hybridizing to each *CYP19A1* exon 1. Subsequently PCR products were subcloned into TOPO cloning vector (Life Technologies) and subjected to direct sequencing. Then, the obtained sequences were examined with BLAST Search (National Center for Biotechnology Information). The presence or absence of promoter-compatible sequences was analyzed with the University of California, Santa Cruz, genome browser (http://genome.ucsc.edu/).

Relative mRNA levels of *CYP19A1* and its neighboring genes

We investigated relative mRNA levels of *CYP19A1* and *DMXL2* as well as those of *CGNL1*, *MAPK6*, *TMOD3*, and *TLN2* involved in the previously reported cryptic inversions (5, 8) in various human tissues. In this experiment, cDNA of SF and LCL were obtained from control males, and the remaining human cDNA samples were purchased from Life Technologies or Takara Bio. Relative quantification of mRNA against *TBP* was carried out using Taqman gene expression assay kit

TABLE 1. Summary of clinical studies in male patients with aromatase excess syndrome[a]

	Present study						Previous studies			
	Family A	Family B	Family C	Family D	Family E	Family F	Family 1	Family 2	Sporadic	
Cases	Cases 1–3	Case 4	Cases 5–6	Cases 7–9	Case 10	Cases 11–18	Two cases[b]	Proband[c]	Patient 1	Patient 2
Mutation type	Duplication	Duplication	Deletion	Deletion	Deletion	Deletion	Inversion	Inversion	Inversion	Inversion
Phenotypic findings										
Gynecomastia	Yes (mild)	Yes (mild)	Yes (moderate)	Yes (moderate)	Yes (moderate)	Yes (moderate)	Yes (severe)	Yes (severe)	Yes (severe)	Yes (severe)
Pubertal defect	Yes (mild)	Yes (mild)	Yes (mild)	No	No	Yes (mild)	N.D.	Yes (mild)	No	N.D.
Short adult height	No	No	N.D.	No	N.D.	No	Yes	N.D.	Yes	N.D.
Spermatogenesis	Preserved	N.D.	N.D.	Preserved	N.D.	Preserved	Preserved	N.D.	N.D.	N.D.
Endocrine findings										
LH (basal)	Normal	Normal	Normal	Normal/low	Normal	Normal/low	Normal	Normal/low	Normal	N.E.
LH (GnRH stimulated)[d]	Low	Normal	High	Normal	Normal	Normal	N.E.	Low	N.E.	N.E.
FSH (basal)	Low	Low	Low	Low	Low	Normal/low	Normal/low	Low	Low	N.E.
FSH (GnRH stimulated)[d]	Low	Low	Low	Low	Low	Low	N.E.	Low	N.E.	N.E.
T (basal)	Normal/low	Normal	Normal/low	Normal/low	Normal	Normal/low	Normal	Normal/low	Low	N.E.
T (hCG stimulated)[e]	N.E.	N.E.	Normal	Normal	Normal	Normal	N.E.	Normal	N.E.	N.E.
E_1 (basal)	High	High	N.E.	High	High	High	High	High	High	N.E.
E_2 (basal)	Normal	High	High	Normal	High	Normal/high	High	High	High	N.E.
E_2 to T ratio	High	High	High	High	High	High	High	High	High	N.E.

E_1, Estrone; N.D., not determined; N.E., not examined.

[a] Detailed actual data are shown in Supplemental Table 1.

[b] A father-son pair.

[c] The sister has macromastia, large uterus, and irregular menses; the parental phenotype has not been described.

[d] GnRH 100 $\mu g/m^2$ (maximum 100 μg) bolus iv; blood sampling at 0, 30, 60, 90, and 120 min.

[e] hCG 3000 IU/m^2 (maximum 5000 IU) im for 3 consecutive days; blood sampling on d 1 and 4.

(assay no. Hs00903411_m1 for *CYP19A1*; Hs00324048_m1 for *DMXL2*; Hs00262671_m1 for *CGNL1*; Hs00833126_g1 for *MAPK6*; Hs00205710_m1 for *TMOD3*; Hs00322257_m1 for *TLN2*; and Hs99999910_m1 for *TBP*). The experiments were carried out three times.

Results

CYP19A1 mRNA levels and aromatase activities

Although relative mRNA levels of *CYP19A1* and catalytic activities of aromatase were grossly similar between LCL of case 3 (family A), case 4 (family B), and case 5 (family C) and those of control subjects, they were significantly higher in SF of case 3 (family A), case 4 (family B), case 9 (family D), and case 10 (family E) than in those of control subjects (Fig. 2).

Sequence analysis of *CYP19A1*

Direct sequencing showed no mutation in *CYP19A1* coding exons 2–10 of the 10 cases examined.

Genome structure analysis

CGH analysis revealed heterozygous cryptic duplications involving most of the *CYP19A1* exons 1 region in cases from families A and B, heterozygous cryptic deletions involving most of *DMXL2* and part of *GLDN* in cases from family C, and heterozygous cryptic deletions involving most of *DMXL2* in cases from families D–F (Fig. 3A). FISH analysis supported the duplications and confirmed the deletions.

Characterization of the cryptic duplications

Aberrant PCR products were obtained with the P2 primer (which amplifies a segment between exon I.1 and exon IIa with the P1 primer) and the P3 primer (which amplifies a segment between exon I.2 and exon I.6 with the P4 primer), and sequencing of the PCR products showed the same tandem duplication involving seven of the 11 exons 1 of *CYP19A1* in cases from families A and B (Fig. 3B). The duplicated region was 79,156-bp long, and the

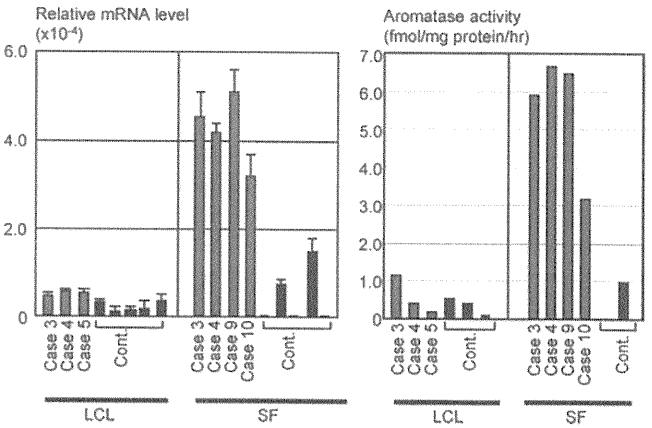

FIG. 2. Relative *CYP19A1* mRNA levels against *B2M* and catalytic activities of aromatase.

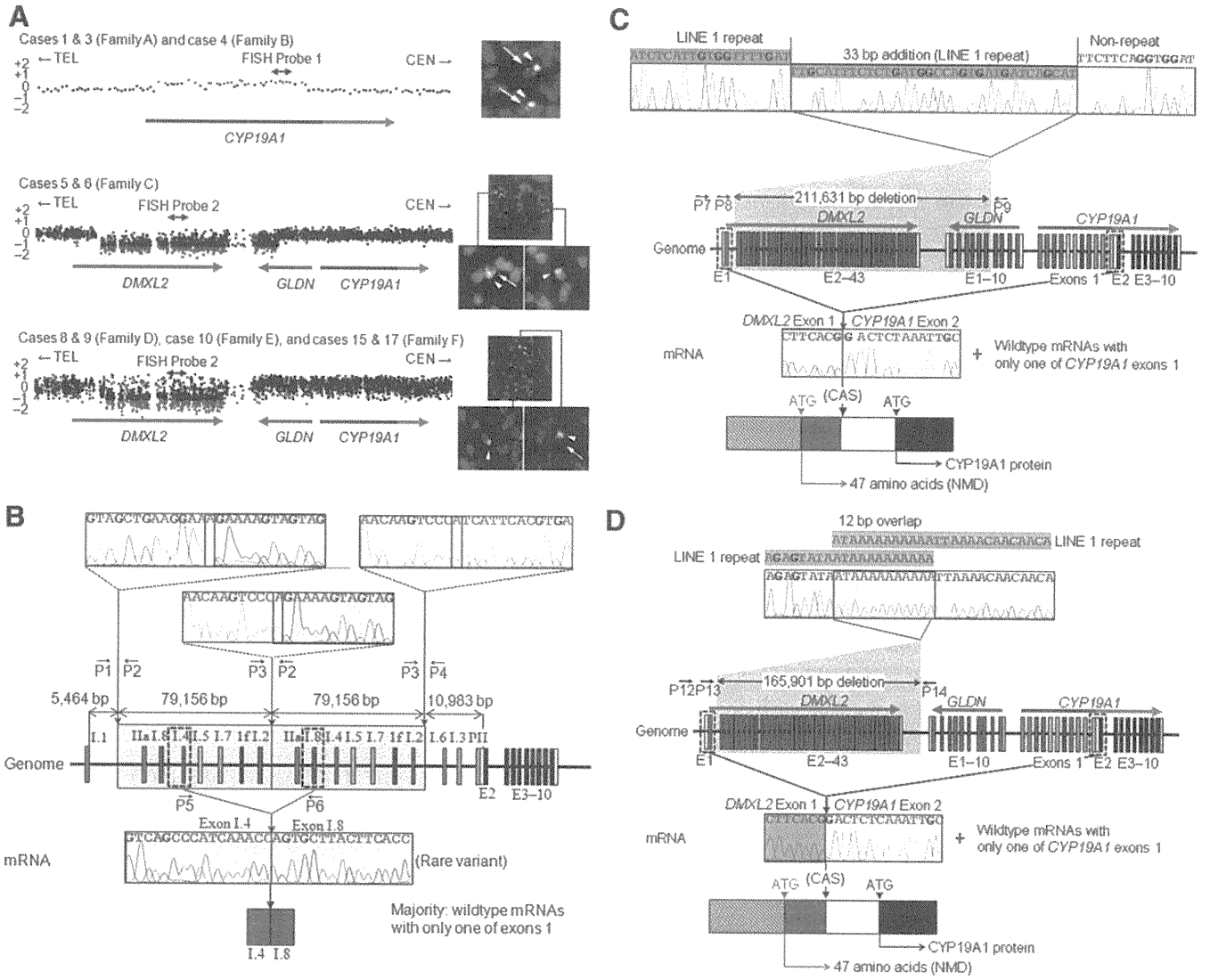

FIG. 3. Summary of molecular studies. For *CYP19A1*, the *dark* and *light blue lines* represent the genomic regions for noncoding exons 1 and coding exons 2–10, respectively. A, Oligoarray CGH and FISH analyses. In CGH analysis, the *black*, *red*, and *green dots* denote signals indicative of the normal, the increased (>+0.5), and the decreased (<−1.0) copy numbers, respectively. In FISH analysis, *two red signals* with an apparently different density are identified in cases from families A and B by FISH probe 1, whereas only a *single red signal* is found in cases from families C–F by FISH probe 2. The *green signals* are derived from the internal control probe. B, Schematic representation of the tandem duplication shared in common by cases 1 and 3 from family A and case 4 from family B. Genome, The junction sequence of the tandem duplication (*yellow boxes*) is shown, together with the original normal sequences at the 5′- and the 3′-ends of the duplicated region. The sequences highlighted with *light green* and *light orange* are identical, and 1 bp (A) is shared at the junction point (highlighted with *light yellow*). mRNA, The sequence of a rare clone is shown. The 3′-end of exon I.4 is connected with the 5′-end of exon I.8. C, Schematic representation of the deletion in sibling cases 5 and 6 from family C. Genome, The junction sequence of the deletion (*a gray area*) is shown. The fusion has occurred between a LINE 1 repeat sequence (*highlighted with blue*) at intron 1 of *DMXL2* and a nonrepeat sequence at intron 4 of *GLDN* and is accompanied by an addition of a 33-bp segment with a LINE 1 repeat sequence. mRNA, The sequence of a rare chimeric gene transcript is shown. *DMXL2* exon 1 consisting of a noncoding region (a *red striped box*) and a coding region (a *red box*) is spliced onto the common acceptor site (CAS) of *CYP19A1* exon 2 comprising an untranslated region (a *white box*) and a coding region (a *black box*). Thus, this transcript has two translation initiation codons (ATG), although the mRNA destined to produce a 47-amino acid protein from the ATG on *DMXL2* exon 1 is predicted to undergo NMD. D, Schematic representation of the deletion shared in common by cases 8 and 9 from family D, case 10 from family E, and cases 15 and 17 from family F. Genome, The junction sequence of the deletion (*a gray area*) is shown. The fusion has occurred between a LINE 1 repeat sequence (*highlighted with blue*) at intron 1 of *DMXL2* and that at a downstream region of *DMXL2*, with an overlap of a 12-bp segment. mRNA, The sequence of a chimeric gene transcript is delineated. The mRNA structure is the same as that described in the legend for Fig. 3C.

fusion occurred between nonrepeat elements with an overlap of one nucleotide.

All the 5′-RACE products (>500 clones) obtained from LCL and SF of case 3 (family A) and case 4 (family B) were found to be associated with a single exon 1, as observed in control materials. However, PCR amplifications for the 5′-RACE products with a variety of combinations of primers hybridizing to each exon 1 and subsequent sequencing of the PCR products revealed the presence of a chimeric clone consisting of exon I.4 at the 5′ side and exon I.8 at

the 3′ side in both LCL and SF (Fig. 3B). Although such a chimeric clone would have been produced by a splice error, this indicated that duplicated exon 1.4 at the distal nonphysiological position functioned as a transcription start site.

Characterization of the cryptic deletions

In cases from family C, long PCR products were obtained with the P7 primer and the P9 primer, and the deletion junction was determined by direct sequencing with the P8 primer (Fig. 3C). The deleted region was 211,631-bp long and involved exons 2–43 of *DMXL2* and exons 5–10 of *GLDN*. The two breakpoints resided within a LINE 1 repeat sequence and a nonrepeat sequence respectively, and a 33-bp segment with a LINE 1 repeat sequence was inserted to the fusion point. In cases from families D–F, long PCR products were obtained by sequential amplifications with the P12 primer and the P14 primer and with the P13 primer and the P14 primer, and an identical deletion was identified by direct sequencing with the P13 primer (Fig. 3D). The deletion was 165,901-bp long and involved exons 2–43 of *DMXL2*. The fusion occurred between two LINE 1 repeat sequences with an overlap of a 12-bp segment.

Sequence analysis of the 5′-RACE products obtained from LCL of cases 5 and 6 (family C) and from SF of case 9 (family D) and case 10 (family E) revealed the presence of a few clones with *DMXL2* exon 1 (2–5%), together with multiple clones with a single wild-type *CYP19A1* exon 1 (Fig. 3, C and D). Such a chimeric mRNA clone was absent from control materials. Furthermore, *DMXL2* exon 1 was found to be accompanied by a promoter-compatible sequence (Supplemental Fig. 2). This indicated a cryptic usage of *DMLX2* exon 1 as an alternative *CYP19A1* transcription start site in cases with deletions. Notably, because of the presence of the translation start codon on *DMXL2* exon 1, mRNAs of the *DMLX2/CYP19A1* chimeric genes are predicted to produce two proteins, *i.e.* CYP19A1 protein and an apparently nonfunctional 47-amino acid protein with a termination codon on *CYP19A1* exon 2, when the translation started from the initiation codons on *CYP19A1* exon 2 and on *DMLX2* exon 1, respectively. Furthermore, mRNA destined to yield the 47-amino acid protein is predicted to undergo nonsense-mediated mRNA decay (NMD) because it satisfies the condition for the occurrence of NMD (12).

Relative mRNA levels of *CYP19A1* and its neighboring genes

CYP19A1 showed a markedly high expression in the placenta and a relatively weak expression in a limited number of tissues including hypothalamus and ovary. By contrast, *DMXL2* was expressed in a range of tissues with some degree of variation as well as *CGNL1*, *MAPK6*, *TMOD3*, and *TLN2* (Fig. 4).

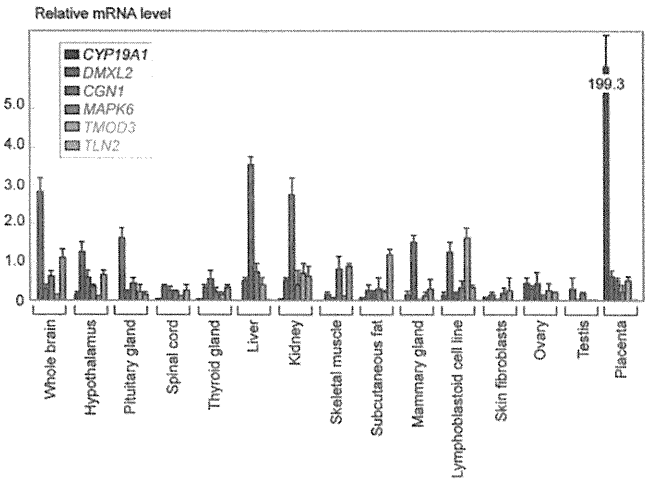

FIG. 4. Expression patterns of *CYP19A1* and the five neighboring genes involved in the chimeric gene formation. Relative mRNA levels against *TBP* are shown.

Discussion

We identified cryptic duplications of the *CYP19A1* promoter region and deletions of the *CYP19A1* upstream region in cases with AEXS. The tandem duplications would have caused *CYP19A1* overexpression because of an increased number of the wild-type transcription start sites. Indeed, because a rare mRNA variant with exon I.4 and exon I.8 was identified, this implies that duplicated exons 1 at the distal nonphysiological position can also function as transcription start sites. Similarly, the deletions would have caused *CYP19A1* overexpression because of a cryptic usage of *DMXL2* exon 1 with a putative promoter function as an extra transcription start site for *CYP19A1*. Indeed, because a few clones with *DMXL2* exon 1 and *CYP19A1* exon 2 were identified, this confirms the formation of a *DMXL2/CYP19A1* chimeric gene. Thus, our results suggest for the first time that duplications of a physiological promoter and deletions of an upstream region can cause overexpression of a corresponding gene and resultant human genetic disease.

Such cryptic genomic rearrangements can be generated by several mechanisms. The tandem duplication in families A and B would be formed by a replication-based mechanism of fork stalling and template switching that occurs in the absence of repeat sequences and is associated with microhomology (13). The deletion in family C is explained by nonhomologous end joining that takes place between nonhomologous sequences and is frequently accompanied by an insertion of a short segment at the fusion point (13).

The deletion in families D–F is compatible with a repeat sequence mediated nonallelic intrachromosomal or interchromosomal recombination (13). Thus, in conjunction with the previously identified four cryptic inversions that are also explainable by fork stalling and template switching or nonallelic recombination (8), genomic sequence around *CYP19A1* appears to harbor particular motifs that are vulnerable to replication and recombination errors.

To date, three types of cryptic genomic rearrangements have been identified in patients with AEXS, *i.e.* duplication type, deletion type (two subtypes), and inversion type (four subtypes) (Fig. 5). Here, although the deletion and the inversion types are associated with heterozygous impairment of neighboring genes (deletion or disconnection between noncoding exon(s) and coding exons), the phenotypes of patients are well explained by excessive *CYP19A1* activity alone. Thus, haploinsufficiency of these neighboring genes would not have a major clinical effect.

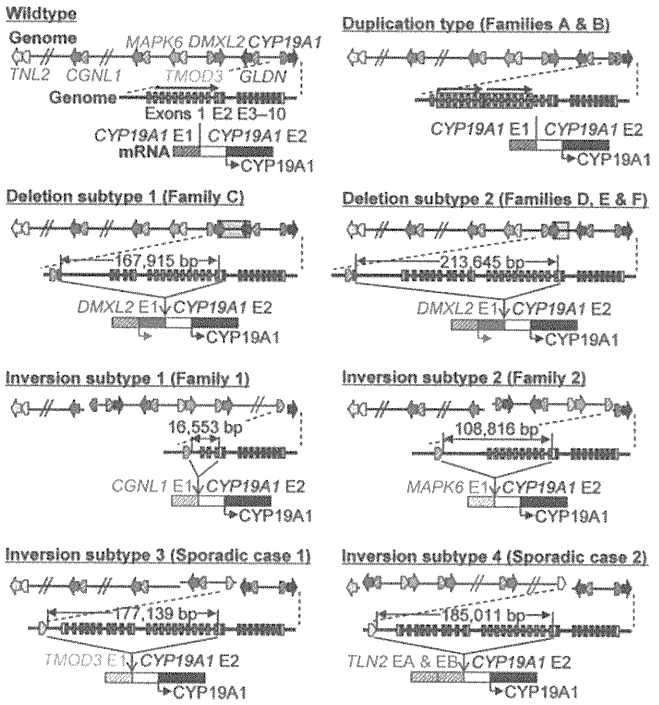

FIG. 5. Schematic representation of the rearranged genome and mRNA structures. The *white* and *black boxes* of *CYP19A1* exon 2 show untranslated region and coding region, respectively (for details, see Supplemental Fig. 1). For the duplication type and the deletion subtypes, see Fig. 3, C and D, for details. For genome, the *striped* and *painted arrows* indicate noncoding and coding exons, respectively (5′→3′). The inverted genomic regions are delineated in *blue lines*. For mRNA, *colored striped boxes* represent noncoding regions of each gene. For *TLN2*, exons A and B correspond to the previously reported exons 1 and 2 (8); because current exon 1 in the public database indicates the first coding exon, we have coined the terms exons A and B for the noncoding exons. The deletion and inversion types are associated with heterozygous impairment of neighboring genes [deletion or disconnection between noncoding exon(s) and the following coding exons]. The inversion subtype 1 is accompanied by inversion of eight of the 11 *CYP19A1* exons 1, and the inversion subtype 2 is associated with inversion of the placenta-specific *CYP19A1* exon I.1.

For the deletion and inversion types, two factors should be considered. One factor is expression patterns of each chimeric gene. In this regard, the five genes involved in the formation of chimeric genes are widely expressed, with some degree of variation (Fig. 4). Furthermore, *in silico* analysis revealed promoter-compatible sequences around exon 1 of *DMXL2*, *CGN1*, *MAPK6*, and *TMOD3* in multiple cell types, although such sequences remain to be identified for noncoding exons of *TLN2* (Supplemental Fig. 2). These findings imply that the chimeric genes show wide expression patterns because expression patterns of chimeric genes would follow those of the original genes.

The other factor is expression dosage of each chimeric gene. In this context, the *DMXL2/CYP19A1* chimeric mRNA was identified only in 2–5% of transcripts from SF, whereas the *CGNL1/CYP19A1* chimeric mRNA and the *TMOD3/CYP19A1* chimeric mRNA accounted for 89–100% and 80% of transcripts from SF, respectively (no data for the *MAPK6/CYP19A1* and the *TLN2/CYP19A1* chimeric genes) (5). This difference is obviously inexplicable by the relative expression level in SF that is grossly similar between *DMXL2* and *TMOD3* and is quite low for *CGNL1* (Fig. 4). In this regard, it is notable that a translation start codon and a following coding region are present on exon 1 of *DMXL2* (Fig. 5). It is likely that *DMXL2/CYP19A1* chimeric mRNA transcribed by the *DMXL2* promoter preferentially recognized the natural start codon on *DMXL2* exon 1 and underwent NMD and that rather exceptional chimeric mRNAs, which recognized the start codon on *CYP19A1* exon 2, were identified by 5′-RACE. By contrast, such a phenomenon would not be postulated for the inversion-mediated chimeric mRNA because of the absence of a translation start codon on the fused exon 1 of *CGNL1* and *TMOD3* (as well as exon 1 of *MAPK6* and exons A and B of *TLN2*) (Fig. 5). For the *CGNL1/CYP19A1* chimeric gene, furthermore, the physical distance between *CGNL1* exon 1 and *CYP19A1* exon 2 is short, and whereas a splice competition may be possible between exon 1 of neighboring genes and original *CYP19A1* exons 1, eight of 11 *CYP19A1* exons 1 including exon I.4 functioning as the major promoter in SF have been disconnected from *CYP19A1*-coding exons by inversion. These structural characters would have also contributed to the efficient splicing between *CGNL1* exon 1 and *CYP19A1* exon 2 (14). In this context, although the *CGNL1/CYP19A1* chimeric gene is associated with functional loss of eight *CYP19A1* exons 1 and the resultant reduction of *CYP19A1* expression in *CYP19A1*-expressing tissues, overall aromatase activity would be increased

by the wide expression of the chimeric gene. These structural properties would primarily explain the difference in the expression dosage of chimeric mRNA between the deletion and the inversion types.

It is inferred, therefore, that the duplication type simply increases *CYP19A1* transcription in native *CYP19A1*-expressing tissues, whereas the deletion and the inversion types cause relatively mild and severe *CYP19A1* overexpression in a range of tissues, respectively. These notions would grossly explain why clinical features of affected males and carrier females and endocrine profiles of affected males are apparently milder in the duplication and the deletion types than in the inversion type and why clinical findings were ameliorated with 1 mg/d of anastrozole in the duplication and the deletion types and with 2–4 mg/d of anastrozole in the inversion type. In addition, the different expression pattern between *CYP19A1* and *DMXL2* may explain, in terms of autocrine and/or paracrine effects, why phenotypic features such as gynecomastia tended to be more severe in the deletion type than in the duplication type under similar endocrine profiles.

Furthermore, several findings are notable in this study. First, a similar degree of FSH-dominant hypogonadotropic hypogonadism is present in the three types, with no amelioration of FSH responses to GnRH stimulation after GnRH priming in two cases with the duplication. This suggests that a relatively mild excess of circulatory estrogens, as observed in the duplication and the deletion types, can exert a strong negative feedback effect on FSH secretion, primarily at the pituitary, as has been suggested previously (15–19). Second, although basal T values appear to be mildly and similarly compromised in the three types, age-matched comparison suggests that T responses to hCG stimulation are apparently normal in the duplication and the deletion types and somewhat low in the inversion type. These data, although they remain fragmentary, would primarily be compatible with fairly preserved LH secretion in the three types and markedly increased estrogen values in the inversion type because T production is under the control of LH (1), and excessive estrogens compromise testicular steroidogenic enzyme activity (20, 21). Lastly, although testis volume appears somewhat small, fertility (spermatogenesis) is normally preserved in the three types. This would be consistent with the FSH-dominant hypogonadotropic hypogonadism because FSH plays only a minor role in male fertility (spermatogenesis) (22). Indeed, males with mutations of *FSHR* encoding FSH receptor as well as mice lacking *FSHB* or *FSHR* can be fertile (23, 24).

The results of this study are contrastive to those of the previous studies. In the previous studies, inversions only have been identified, and each inversion is specific to each family or patient (8). By contrast, in this study, the identical duplication was found in two Japanese families A and B, and the same deletion (subtype 2 in Fig. 5) was shared by three Japanese and one Caucasian families D–F, despite apparent nonconsanguinity. This may be explained by assuming that patients with severe phenotype were preferentially examined in the previous studies, whereas those with the AEXS phenotype were analyzed in this study without ascertainment bias. Furthermore, because phenotypes are milder in the duplication and the deletion types than in the inversion type, this may have permitted the spread of the duplication and the deletion types, but not the inversion type, as the founder abnormalities. This notion predicts that the duplication and the deletion types would be identified by examining patients with mild AEXS phenotype.

In summary, the present study shows that AEXS can be caused by duplications of the physiological promoters and microdeletions of the upstream regions of *CYP19A1* and that phenotypic severity is primarily determined by the tissue expression pattern of *CYP19A1* and the chimeric genes and by structural properties of the fused exons. Most importantly, the present study provides novel models for the gain-of-function mutations leading to human genetic disease.

Acknowledgments

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日本小児科学会雑誌　115巻1号　1～4（2011年）

総　説

日本の性分化疾患の実情

I. 性分化疾患の実態調査結果

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大　山　建　司

キーワード：性分化疾患，実態調査，性腺

はじめに

性分化の異常を有する症例は，疾患の性質上また社会通念上，隠蔽される傾向にあり，長くその実態は明らかにされてこなかった．しかし近年，性分化機構の解明が飛躍的に進歩し，疾患の原因が明らかになるにつれて，疾患への関心が高まり，患者が抱える様々な問題点も注目されるようになってきた．このような社会環境の変化の中で，我が国でも従来使用されてきた疾患名の見直し，性分化疾患の実態把握，社会的緊急性が高い新生児期の初期対応が日本小児内分泌学会を中心に検討されている．本稿で述べる性分化疾患の実態調査，初期対応は表1に示す組織が合同で行ったものである．

疾患名の削除・変更

従来，インターセックス（間性），半陰陽（雌雄同体），仮性半陰陽等と呼ばれていた疾患は，総称して性分化異常症と呼ばれていた．しかしこれらの用語には倫理的問題や侮蔑的な意味合いを含むものもあり，命名法の再検討が必要と考えられるようになった[1)2)]．2006年性分化異常症の専門家による国際会議が開催され，新たな英文病名が提唱された．その結果，Disorders of Sex Differentiation は Disorders of Sex Development（DSD）に変更された．この国際会議におけるコンセンサスの詳細は緒方が報告している[3)]．これに伴い，2009年第43回日本小児内分泌学会総会で，日本語病名を性分化異常症から性分化疾患に変更することを決定し，周知していくこととなった．

性分化疾患は，「染色体，性腺，または解剖学的性が非定型である先天的状態」と定義されている．一方，性同一性障害は定まった定義はできていないが，一般的には，生物学的に男女いずれかの身体的形状を有するにもかかわらず，性自認が生物学的性と一致しない状態とされている．ここでいう性自認は，染色体，性腺，ホルモン，内性器，外性器，ジェンダー，性役割・性指向のいずれからも独立していることが条件とされている．性分化疾患に伴う性同一性障害は一般的な性同一性障害とは分けて分類されている．

性分化疾患の用語の中で，今後使用すべきではないと考えている疾患名としては，前述したインターセックス（間性），半陰陽（雌雄同体），仮性半陰陽以外に，類宦官症（低または高ゴナドトロピン性類宦官症），睾丸性女性化症，副腎性器症候群が挙げられている．真性半陰陽は卵精巣性性分化疾患，男性または女性仮性半陰陽は，新たな用語となる46, XY または46, XX性分化疾患に含まれる．卵巣に対応する呼称として睾丸は精巣に改める（停留精巣，精巣形成不全，精巣機能低下症等）．

性分化疾患の実態調査

我が国における性分化疾患の実態はこれまで，一部の疾患を除いて明らかにされていなかった．今回，日本小児内分泌学会性分化委員会（委員長：大山建司）と厚生労働省難治性疾患克服研究事業（研究代表者：緒方勤）が合同で，日本小児内分泌学会と日本小児泌尿

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表1　性分化疾患の実態調査，初期対応の検討を行った組織

1. 日本小児内分泌学会性分化委員会
（大山建司，緒方勤，有阪治，堀川玲子，位田忍，立花克彦，向井徳男，佐藤清二，木下英一）
2. 厚生労働科学研究費補助金，難治性疾患研究事業
1）性分化疾患の実態把握と治療指針作成班（緒方勤，大山建司，堀川玲子，藤枝憲二，有阪治，島田憲次，野々村克也）
2）チトクローム P450 オキシドレダクターゼ異常症の実態把握と治療指針作成班（深見真紀）
3）副腎ホルモン産生異常に関する調査研究班（藤枝憲二）

表2　性腺の発生分化に関連する性分化疾患

	小児科領域		小児泌尿器科領域		合計	
疾患名	確診	疑い	確診	疑い	確診	疑い
性腺形成障害（原因不明）	10	18	1	3	11	21
卵精巣性性分化疾患（真性半陰陽）	26	2	22	0	48	2
XY 性腺形成異常症	7	2	2	1	9	3
XX 性腺形成異常症	3	1	0	0	3	1
XX 男性（SRY +）	4	2	5	0	9	2
XX 男性（SRY −）	6	0	3	0	9	0
XY 女性（SRY 異常あり）	6	0	0	0	6	0
XY 女性（SRY 異常なし）	7	1	0	0	8	1
上記以外の性染色体異常（ターナー症候群を除く）	77	2	21	0	98	2
WT1 異常症	20	3	10	0	30	3
SF1 異常症	6	0	0	0	6	0
DAX1 異常症	38	3	0	1	38	4
SOX9 異常症	3	1	1	0	4	1
DHH（desert hedgehog）異常症	0	0	0	0	0	0
9p 欠失	1	0	0	0	1	0
ARX 異常症（XLAG）	0	0	0	0	0	0
ATRX	5	1	0	0	5	1
ATR-16	0	0	0	0	0	0
10q25-Cter 欠失	1	1	0	0	1	1
WNT4/RSPO1 過剰発現	0	0	0	0	0	0
WNT4 異常症	0	0	0	0	0	0
RSPO1 異常症	0	0	0	0	0	0
高次脳機能障害を伴う性分化異常症	2	1	0	0	2	1
小脳失調を伴う性分化異常症	4	0	0	0	4	0
合計	226	38	65	5	291	43

器科学会の全学会員 1,911 名を対象として調査を行った．集計は施設毎に行った．施設数は小児内分泌 326 施設，小児泌尿器 166 施設であった．調査に当っては従来一般的に使用されている疾患名を併記した．調査結果を表 2〜5 に示す．生殖器奇形は，出生 4,500 人に 1 例の頻度と推定されている．今回の調査対象からターナー症候群は除いた．ターナー症候群は出生女児 2,500 人に 1 人の頻度と推定されている[4]．

性腺の発生・分化に関連する性分化疾患（表 2）では，確診例の中で卵精巣性性分化疾患 3 例，上記以外の性染色体異常 3 例，WT1 異常症 2 例が重複例と推測されたため，確診例総数は 283 例，疑い例 43 例である．確診例の中では，卵精巣性性分化疾患，DAX1 異常症，WT1 異常症が多数を占めた．卵精巣性性分化疾患の染色体核型は 46, XX が 60% 以上と頻度が高く，次いで 46, XX/46, XY キメラが 20%，46, XY は 10% 以下と

表3 46, XY性分化疾患

	小児科領域		小児泌尿器科領域		合計	
疾患名	確診	疑い	確診	疑い	確診	疑い
原因不明の46XY性分化異常	46	53	11	2	57	55
GnRH受容体異常症	0	0	0	2	0	2
GPR54異常症	0	0	0	0	0	0
LH異常症	0	0	0	1	0	1
FSH異常症	0	0	0	1	0	1
LH受容体異常症	2	1	1	0	3	1
アンドロゲン受容体異常症	43	19	20	9	63	28
Smith-Lemli-Opitz症候群	3	0	2	0	5	0
Aaskog-Scott症候群	3	3	0	0	3	3
Robinow症候群	2	3	1	0	3	3
胎児期精巣退縮症候群	22	10	51	9	73	19
ミュラー管遺残症候群	2	2	4	2	6	4
尿道下裂（CXorf6, BMP4, BMP7, HOXA4, HOXB6, HOXA13異常）	3	0	0	0	3	0
合計	126	91	90	26	216	117

表4 46, XX性分化疾患

	小児科領域		小児泌尿器科領域		合計	
疾患名	確診	疑い	確診	疑い	確診	疑い
原因不明の46XX性分化異常	1	1	1	0	2	1
卵巣形成不全，無形成	7	0	0	0	7	0
子宮形成不全，無形成 Mayer-Rokitansky-Kuster（MRK）症候群	8	3	3	6	11	9
子宮形成不全，無形成 MURCS（Mullerian, renal, cervical spine）	0	2	0	0	0	2
子宮形成不全，無形成 MODY5に合併	0	0	0	0	0	0
子宮形成不全，無形成その他	2	1	1	0	3	1
腟形成不全 McKusck-Kaufman症候群（BBS6）	0	0	1	0	1	0
腟形成不全 hannd-foot-genital症候群（HOXA13）	0	0	0	0	0	0
腟形成不全その他	3	1	2	2	5	3
合計	21	8	8	8	29	16

報告されている[5]. 小脳失調を伴う性分化疾患が4例報告され，新たな疾患群となる可能性がある．表2に「上記以外の性染色体異常」が100例報告されており，詳細は不明だがクラインフェルター症候群が含まれている可能性はある．

46, XY性分化疾患（表3）ではアンドロゲン受容体異常症4例，LH受容体異常症1例が重複例と推測された．全体で確診例211例，疑い例117例である．泌尿器科領域では胎児期精巣退縮症候群，小児科領域ではアンドロゲン受容体異常症が多数を占めていた．両側精巣の胎児期精巣退縮症候群は出生男児20,000人に1人と報告されている[6]. アンドロゲン受容体異常症は出生男児20,000～99,000人に1人と報告されているが[7][8]，遺伝子診断が普及してきたため，発生頻度は今後増加する可能性がある．

46, XX性分化疾患（表4）では，MRK症候群1例，腟形成不全その他の1例が重複していると思われる．重複例を除くと確診例27例，疑い例16例で，総数は43例である．46, XX性分化疾患の大部分は後述する先天性副腎過形成症が占める．それ以外の46, XX性分化疾患は，新生児期に外性器異常を伴わない症例（完全女性型）が多く存在するため，思春期以降に婦人科を受診する可能性も高く，そのような症例は婦人科を調査対象としていない今回の調査からは漏れている可能