such as cosmetics. DEP, DMP, and DBP are the phthalate esters used in personal care products as well as in plastics and others. Considering this use, people are likely to be exposed to DMP, DEP, and DBP, especially via dermal absorption through the usage of cosmetics. Duty et al. [38] reported significant association between urinary MEP concentration and frequency of usage of personal care products. The MOE estimation did not include dermal exposure, which might have resulted in underestimation of exposure. The contribution from personal care products has to be quantitatively estimated for Japanese population to fully assess exposure level of phthalate esters.

On the contrary, our estimated intake of DEHP was lower than the MOE estimation (Fig. 2). In 2000, the Ministry of Health and Welfare of Japan restrained DEHP use in polyvinyl chloride (PVC) gloves because it was suspected that handling of foods with PVC gloves contaminated foods with DEHP. After this regulation, DEHP concentration in food decreased and average daily intake of DEHP decreased from 519 μg/day in 1999 to 160 μg/day in 2001 [39]. Since the MOE used DEHP concentration in food reported in 1998 for intake estimation, this might have resulted in overestimation of daily DEHP intake level. When we used newer exposure information of DEHP from food [39] and indoor air [40], calculated DEHP intake was 3.33 μg/kg per day, which was consistent with the present estimations from urinary metabolite concentration (1.1–2.2 μg/kg per day). This consistency may support our notion that DEHP exposure from food had recently decreased substantially in Japan.

Exposure levels to phthalate esters in 50 pregnant women

As shown in Table 3, our estimates of daily intakes of phthalate diesters were lower than TDIs set by the EFSA based on reproductive and developmental effects to offspring in animal experiment. Swan et al. [8] observed relationships between prenatal phthalate exposure and anogenital distance (AGD) of male infants and indicated that humans might be more sensitive to prenatal exposure to phthalate esters than were rodents. The phthalate exposure levels in this study were similar to those in US pregnant women who had male infants with reduced AGD by prenatal phthalate exposure in the Swan et al. study [8] (Table 3). Therefore the present phthalate ester exposure levels might not completely be effect-free, although the intake levels of phthalate esters in this study were lower than TDI values set by the EFSA.

The present study revealed the fact that metabolites of various phthalate esters are detected in urine samples collected from Japanese pregnant women, showing that they were exposed to phthalate esters on a daily basis. It is not clear whether this exposure level of Japanese pregnant women is safe enough for their offspring, though the level was less than TDIs. Further studies are needed to reveal the toxicity of phthalate esters to fetus and other sensitive subpopulations. Exposure monitoring of sensitive subpopulation to phthalates has to be continued as well.

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原　著

日本人小児の多環芳香族炭化水素類曝露評価

森　拓哉[*1], 吉永　淳[*1], 河原　純子[*2], 溝井　美穂[*3], 安達　修一[*3]

[*1] 東京大学大学院新領域創成科学研究科
[*2] 国立環境研究所
[*3] 相模女子大学大学院栄養科学研究科

Assessment of Exposure to Polycyclic Aromatic Hydrocarbons in Japanese Children

Takuya MORI[*1], Jun YOSHINAGA[*1], Junko KAWAHARA[*2], Miho MIZOI[*3] and Shuichi ADACHI[*3]

[*1]*Department of Environmental Studies, University of Tokyo*
[*2]*National Institute for Environmental Studies*
[*3]*Sagami Woman's University*

Abstract **Objectives: To estimate the level of exposure to polycyclic aromatic hydrocarbons (PAHs) in Japanese children by urinary metabolite analysis and the possible contribution of soil ingestion and environmental tobacco smoke (ETS) to PAHs exposure.**

Methods: Spot urine samples and questionnaire data were collected from 107 kindergarten children (3–6 yrs) and their mother. The urinary concentration of 1-hydroxypyrene (1-OHP), a biomarker of PAHs exposure, was measured using a high performance liquid chromatography-fluorescence detector.

Results: The geometric mean (GM) of urinary 1-OHP concentrations in children was 0.065 μmol/mol-cre (geometric standard deviation=1.88). Parental smoking and time of playing outside (surrogate of soil exposure level) did not increase urinary 1-OHP level. Maternal urinary 1-OHP concentration correlated with, whereas GM (0.038 μmol/mol-cre) was significantly lower than, the urinary 1-OHP concentration in children. The latter might be attributable to greater amount of food intake per body weight for children than for adult.

Conclusions: The contribution of ETS and soil ingestion to PAHs exposure seemed to be small and thus they cannot be the major source of PAHs in Japanese children.

Key words: biomarker (バイオマーカー), exposure assessment (曝露評価), Japanese children (日本人小児), polycyclic aromatic hydrocarbons (多環芳香族炭化水素類), urine (尿)

I. はじめに

多環芳香族炭化水素類 (PAHs) は2つ以上の縮合芳香環をもつ多くの種類を含む化合物類であり，有機化合物の不完全燃焼により非意図的に発生する (1)。PAHs のうちいくつかはヒトに対して発癌性が高いと各機関 (2, 3) で位置付けられており，IARC による発癌リスク評価において benzo[a]pyrene (B[a]P) はグループ1 (「ヒトに対し発がん性あり」) に，benzo[a]anthracene, dibenzo[a,h]anthracene はグループ2A (「ヒトに対しおそらく発がん性あり」) に，benzo[b]floranthene (B[b]F) を含む9種はグループ2B (「ヒトに対し発がん性を持つ可能性あり」) に分類されている。また，米国の毒性物質登録機関 (ATSDR) が公衆衛生に重大な影響を与える物質に優先順位をつけて作成している "Priority List of Hazardous Substances" によれば，2007年時点で PAHs は8位，B[a]P は9位，B[b]F は10位とされており (4)，その有害性について注目すべき物質である。

1997年に採択された「マイアミ宣言」をきっかけとし，近年では化学物質の毒性に脆弱な小児の健康リスクを考慮した化学物質の有害性評価が求められている。最近になって，胎児期の PAHs 曝露による出生体重低下 (5) や

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Reprint requests to: Jun YOSHINAGA
Department of Environmental Studies, University of Tokyo, Kashiwa-noha 5-1-5, Kashiwa, Chiba 277-8563, Japan
TEL/FAX: +81(4)7136-4716
E-mail: junyosh@k.u-tokyo.ac.jp

認知発達低下 (6) といった影響との関連の示唆が報告されており，PAHs の発達毒性にも注目していく必要があると考えられる。そのために小児を対象とした曝露評価および影響評価の充実が必要である。

PAHs 曝露レベルの評価方法として，PAHs の一つである pyrene の代謝産物である 1-hydroxypyrene (1-OHP) の尿中排泄量を指標としたバイオマーカー法が広く用いられている。pyrene は PAHs の主要な一成分であり，総 PAHs 曝露量と尿中 1-OHP 濃度の間に相関が見られることから，尿中 1-OHP 濃度によって PAHs の曝露評価が可能とされ (7-9)，小児の曝露評価に尿中 1-OHP 濃度を使用した例が数多くある (10-12) が，わが国の小児については，Kanoh ら (13) による報告があるのみである。彼らは大気汚染レベルの異なる地域に住む小児の尿中 1-OHP 濃度について調査しており (地域・季節により尿中 1-OHP 濃度は約 10-20 ng/dl)，大気汚染の少ない東京都東大和市に比べて東京都目黒区・板橋区の尿中 1-OHP 濃度が高いことから，小児の PAHs 曝露に大気汚染の寄与が大きいことを報告している。しかしながら，燃料の良質化・燃焼機構の高効率化・排気機構の高度化によって，わが国の PAHs による大気汚染レベルが低下するにしたがい (14)，現在では大気からの曝露の相対的寄与率も低下していると考えられる。

職業的な曝露のない一般公衆の PAHs 曝露源として，汚染大気以外に食物とタバコが知られている (11)。後者については小児の場合，受動喫煙が問題となる可能性があり，Environmental tobacco smoke (ETS) が尿中 1-OHP 濃度の増加に寄与しているとの報告も挙がっている (10, 15, 16)。さらに，最近になって化学物質曝露源として土壌が注目されている。特に小児の場合はほふく や砂遊び，発達期の手や物を口に入れる特有の行動 (マウジング) によって，成人より多くの土壌を摂取しているとされ，曝露評価において土壌の直接摂取由来の曝露推定は不可欠となっている。

本研究では，既往研究において広く用いられている尿中 1-OHP 濃度を指標として，現在の日本人小児の PAHs 曝露レベルを，土壌や ETS への曝露との関連から評価することとした。

II. 対象と方法

1. サンプリング

調査対象者は神奈川県 S 市某幼稚園に通う幼児 (3-6 才) とその母親 107 組とし，2007 年 10 月に尿のサンプリングを行った。事前にサンプリング用の採尿カップとポリプロピレン製ボトル，質問票を対象者 (保護者) に配布し，期間中任意の朝の，その日最初のスポット尿を保護者により採尿コップに採取した後，ポリプロピレン製ボトルに移してもらった。母子の尿サンプルは，サンプリングの朝，幼稚園内に用意したアイスボックスに，回答済みの質問票とともに提出してもらった。回収した尿サンプルはただちに 2 本のポリプロピレン製遠沈管にほぼ 10 ml ずつ分注し，−20°C で冷凍保存した。質問票の内容は，①母子の性別や年齢などの基本的な情報，②家庭内の喫煙者の有無，③屋外で遊んだ時間，とした。

なお，本研究は調査に関与した各組織の倫理審査委員会の承認を得たものである。また事前に小児保護者らに本研究に関する説明文書を配布し，書面にて同意を得られた母子を調査対象者とした。

2. 方法

尿試料は 5°C にて解凍した後に 5 ml を遠沈管にとり，0.1 M 酢酸ナトリウム緩衝溶液 10 ml, β-glucuronidase/aryl sulfatase (type H-2, Sigma, USA) 15 μl を加え，37°C で 2 時間インキュベートして 1-OHP のグルクロン酸及び硫酸抱合体の加水分解を行なった。固相抽出用の Sep-PaK C18 カラム (Waters, USA) は事前にメタノール (高速液体クロマトグラフ用，関東化学，日本) 5 ml と超純水 10 ml でコンディショニングしておき，試料溶液を通液させた。40% メタノール 10 ml でカラムを洗浄し，メタノール 10 ml を通液させて 1-OHP を溶出した。溶出液を窒素気流下で 39°C に加温，溶媒を蒸発・乾固したのちに，メタノール 0.5 ml で再溶解し，検液として高速液体クロマトグラフィー—蛍光検出器 (HPLC-FL, 日立ハイテク社製，東京) での測定に供した (励起／発光波長 :242/388 nm)。HPLC カラムは Waters PAH C18 (4.6×250 mm, Waters, USA) をカラムオーブンで 31°C に保って用い，移動相はメタノール：水＝70:30, 流量 1 mL/min とした。なお試料注入量は 10 μL とした。

本測定においては，S/N＝3 より算出した検出下限 0.0034 ng/ml (尿中 1-OHP 濃度)，併行精度 3.2-6.2%，再現性 6.1%，回収率 105±4.4% (尿中 1-OHP 濃度 0.5 ng/ml 程度＋添加濃度 0.5 ng/ml) であった。精度管理として予め作成した x-R 管理図を元に評価しながら分析をすすめるとともに，German External Quality Assessment Scheme 40 (2007) による試験所間比較に参加して測定の真度を確認した。

また本調査ではサンプルとしてスポット尿を用いたため，クレアチニン濃度を用いて尿量補正を行なった。クレアチニンの測定には，クレアチニンテストワコー (和光純薬工業(株)，大阪) を用い，Jaffé 法に基づいて行った。

統計解析は，クレアチニン補正した尿中 1-OHP 濃度を対数変換したのち，男女差の検定には t 検定，年齢群や ETS，外遊び時間との関連解析には一元配置分散分析 (ANOVA)，年齢との相関分析はピアソンの相関分析を SPSS ver. 12J を用いて行った。

III. 結　　果

1. 尿中 1-OHP 濃度

被験者となった小児 107 名の尿中 1-OHP 濃度は，濃度範囲 0.018-1.25 μmol/mol-cre, 幾何平均 (GM) 0.065 μmol/

表1　本研究と既往研究における尿中1-OHP濃度［μmol/mol-cre］

調査国	小児		母親（成人）		引用文献
	幾何平均	最小-最大	幾何平均	最小-最大	
日本	0.065	0.018-1.25	0.038	0.011-0.236	本研究
	——	——	0.046	0.011-0.147	(17)
	——	——	0.011	0.004-0.028	(18)
	——	——	0.06	0.01-0.06	(19)
	(平均 :0.079-0.108)*		—	—	(13)
アメリカ	0.052	—	0.027	—	(11)
チェコ	0.075	0.010-0.76	—	—	(12)

* Kanohらはクレアチニン未補正・幾何平均不算出のため，示されていた平均値（約0.10-0.20ng/ml）を本研究の平均クレアチニン濃度で補正した算術平均を示す。

mol-cre，幾何標準偏差（GSD）1.88であった。図1にヒストグラムで示したように，107名の対象小児のなかで1例のみ飛び離れて大きな値（1.25μmol/mol-cre）が見られた。以下の統計解析の際にはこの値を含む場合と除外した場合の2通り行ったが，統計解析結果の有意性が異なることはなかった。一方，小児の母親107名の尿中1-OHP濃度範囲は0.011-0.236μmol/mol-cre，GM 0.040μmol/mol-cre，GSD 1.85であった。濃度分布は母子共に対数正規分布であった（図1，2）。表1には，本研究で得られた尿中1-OHP濃度と文献値（11-13, 17-19）とを比較した。本研究における小児と母親の尿中1-OHP濃度は，国内外の既往研究とほぼ同じレベルであった。Kanohら（13）は尿中1-OHP濃度（ng/ml）をクレアチニン未補正，算術平均で結果を報告しているので，彼ら尿中1-OHP濃度の算術平均値を本研究で得られた小児のクレアチニン濃度の平均値（0.82mg/ml, n＝107）で補正した値を示した。地域ごとに，板橋区0.108，目黒区0.102，東大和市0.079μmol/mol-creとなった。Kanohらの値は算術平均値であるということを考慮すると，本研究でえられたGMの0.065μmol/mol-creとほぼ同じ濃度レベルであると推定された。なお，本研究の尿中1-OHPの算術平均値は0.085μmol/mol-creである。

対象児童の男女別の尿中1-OHP濃度についてt検定を行なったが，有意な差はなかった。一方，年齢別の尿中1-OHP濃度については，弱い有意な負の相関が見られた（$p<0.01$，$r=-0.26$）。なお，小児の男女間で年齢構成に有意な差はみられず（χ^2検定，$p=0.52$），年齢ごとの男女差も見られなかった（t-検定）。

2. ETSとの関連

アンケートによる生活調査から，小児を①喫煙者のいない家庭（n＝63），②母親が喫煙者である家庭（n＝6），③母親以外の成人が喫煙者である家庭（n＝37），の3群に分けたところ，各群の尿中1-OHP濃度のGMは表2に示すとおり0.064，0.069，0.068μmol/mol-creで，3群間に有意な差は見られなかった（ANOVA，$F=0.139$，$p=0.87$）。また尿サンプリング前日に家庭内および外出

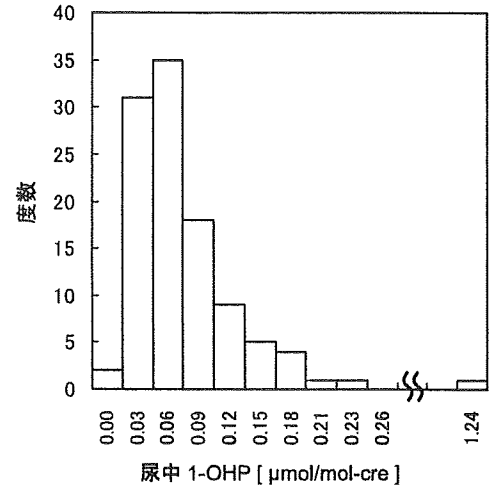

図1　小児の尿中1-OHP濃度分布。GM 0.065μmol/mol-cre, GSD 1.88（n＝107）。

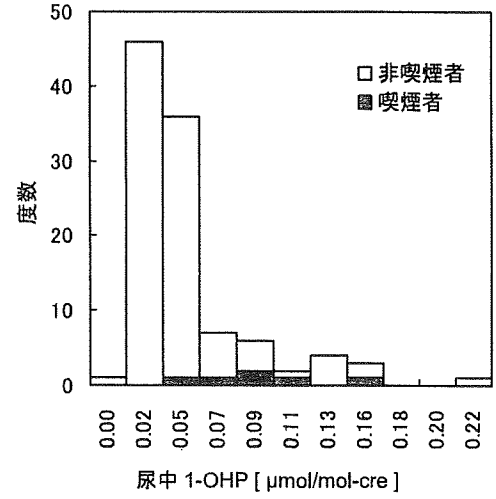

図2　母親の尿中1-OHP濃度分布。全母親：GM 0.040μmol/mol-cre, GSD 1.85（n＝107），喫煙者：GM 0.086, GSD 1.55（n＝6），非喫煙者：GM 0.036, GSD 1.81（n＝100）。喫煙者 vs 非喫煙者（t検定）：$p<0.01$。

表2 家族内の喫煙者の有無と小児の尿中1-OHP濃度

	喫煙者なし	母親が喫煙	母親以外が喫煙	ANOVA	
n	63	6	37	F	p
尿中1-OHP濃度 (μmol/mol-cre)*	0.064 (1.7)	0.069 (1.6)	0.067 (2.3)	0.139	0.870

* 幾何平均 (幾何標準偏差)

先でETSへの曝露が母親によって観察された群 (n=17, GM 0.074 μmol/mol-cre) と観察されなかった群 (n=89, GM 0.065 μmol/mol-cre) についてもt検定を行なったが, 有意な差は見られなかった (p=0.41)。

母親のタバコ煙への曝露については, 喫煙者 (n=6) と非喫煙者 (n=100) に分けると喫煙者の尿中1-OHP濃度は有意に高かった (GM 0.086 vs 0.038 μmol/mol-cre, p<0.01)。同居者に喫煙者のいる非喫煙者 (n=37) といない非喫煙者 (n=63) では有意な差はみられなかった (GM 0.038 vs 0.039 μmol/mol-cre, p=0.78)。

なお以上は喫煙関係のデータが得られなかった (無回答) 1名の母親を除外してn=106で求めたものである。

3. 土壌接触頻度との関連

屋外で遊ぶ時間が多いほど, 土壌曝露の機会が増えると考え, 質問票によって調査した「屋外で遊ぶ時間」が小児の尿中1-OHP濃度の変動要因となっているか解析を行なった。

小児がサンプリング前日に屋外で遊んだ時間は0時間 (n=38), 0-1時間 (n=41), 1-2 (n=10), 2-3時間 (n=7) であった。小児らがサンプリング前日に屋外で遊んだ時間と尿中1-OHP濃度について一元配置分散分析を行ったところ, 有意な関連はみられなかった (F=1.787, p>0.05, 図3)。またサンプリングまでの一週間に屋外で遊んだ時間は0-5時間 (n=36), 5-10時間 (n=33), 10-15時間 (n=17), 15-20時間 (n=8), >20時間 (n=7) であり, これと尿中1-OHP濃度についても同様の解析を行ったところ, 有意な関連は見いだせなかった (F=2.127, p>0.05)。

小児のマウジング頻度については, 母親の観察に基づき, 「よくみられる」(n=17), 「ときどきみられる」(n=49), 「まったくみられない」(n=41), の3群に分け, これら3群の尿中1-OHP濃度についてANOVAを行なったが, 有意な変動はみられなかった (F=1.295, p>0.05, 表3)。

4. 母親の尿中排泄量との相関

母子の尿中1-OHP濃度を比較するとGM 0.038と0.065 μmol/mol-creであり, 小児のほうが約2倍高い値を示した。母子のペアごとに比較すると, 小児の方が有意に高い値となった (対応のあるt検定, p<0.01, 表1)。また母子の尿中1-OHP濃度の対数値についてピアソン相関分析を行ったところ有意な正の相関がみられた (r=0.45, p<0.01, 図4)。

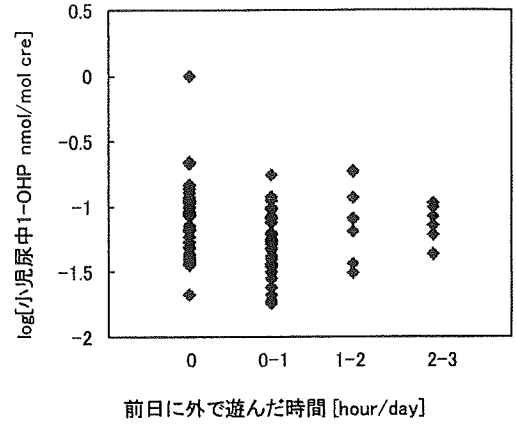

図3 採尿前日の土壌との接触時間と小児の尿中1-OHP濃度。F=1.787, p>0.05 (ANOVA)。

IV. 考 察

1. 本研究における尿中1-OHP濃度について

本研究における尿中1-OHP濃度については, 表1に示したように, 国内外の一般公衆における近年の報告値とほぼ同じレベルであり, 本研究の対象者のPAHs曝露量はごく一般的なレベルであると考えられる。近年の報告では一般公衆のPAHs曝露において大気の寄与はほとんどなく, 主に食事が寄与していると言われている (18, 20)。

既往研究において唯一日本人小児の尿中1-OHP濃度を調査しているKanohら (13) の報告では, 1988-1989年の大気汚染の少ない住宅地 (東京都東大和市) と相対的に大気汚染の強い都市部 (目黒区・板橋区) での尿中1-OHP濃度の差から, 小児のPAHs曝露には大気汚染の寄与があると述べている。本研究では調査地域として都心から直線距離でおよそ30 km離れた住宅地域を対象としたが, Kanohらの目黒・板橋区では小児の尿中1-OHP濃度が推定算術平均0.10-0.11 μmol/mol-cre相当と本研究よりやや高値であり, 比較的似た生活環境であると考えられる東大和市では算術平均推定0.079 μmol/mol-cre相当で本研究より若干低い, あるいはほぼ同レベルといえる。またKanohらが調査した1988-1989年平均の大気中B[a]P濃度は東大和市0.89 ng/m³, 板橋区1.78 ng/m³, 目黒区1.80 ng/m³程であった (13)。一方, 本研究の対象である2007年度の神奈川県一般環境の大気中平均B[a]P濃度は0.16 (0.10-0.30) ng/m³であった (21)。大気中の

表3　マウジング行動と小児の尿中1-OHP濃度

	よくみられる	ときどきみられる	まったくみられない	ANOVA	
n	17	49	41	F	p
尿中1-OHP濃度 (μmol/mol-cre)*	0.082 (1.5)	0.062 (2.1)	0.064 (1.8)	1.295	0.278

*幾何平均 (幾何標準偏差)

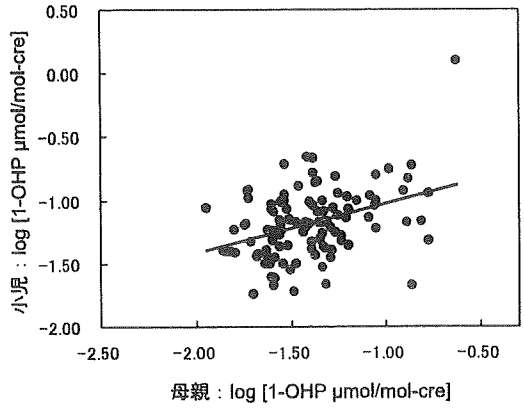

図4　母子の尿中1-OHP濃度の相関。r=0.45, p<0.01, 回帰式 y=0.393x−0.634。

B[a]P濃度を比較してみると，2007年の神奈川県は1988年の東大和の1/5，目黒・板橋の1/10と低下している。それにもかかわらず，尿中1-OHP濃度はあまり変化しておらず，東大和については本研究結果とほぼ同値である。これはPAHsの総曝露量に対して大気の寄与が小さいために，2007年神奈川と1988年東大和の大気寄与の差が総曝露量の変動に隠れてしまったためと考えられ，PAHsの主要な曝露源は当時も今も大気ではないと考えられる。

2. ETSの寄与

一般に喫煙者の尿中1-OHP濃度は非喫煙者に比較して高いことがよく知られており，本研究の対象保護者(母親)でも同様の結果が見られた。本研究では保護者に小児や本人のETS曝露に関わる質問を行い，その集計結果をもとに対象者を群分けして検討したが，小児の尿中1-OHP濃度には有意な変動はみられなかった (表2)。既往の研究では，ETS曝露によって小児の尿中1-OHP濃度が高くなることが示されている (10, 15, 16) が，本研究ではETS曝露が小児や母親のPAHs曝露に大きな影響を与えていないという結果となった。

調査対象において，喫煙者のいる44家庭中32家庭が子供の前での喫煙を一切控えているなど，分煙意識が高かったことから，家庭内の喫煙者の有無だけではETS曝露の実態をよく反映していない可能性がある。また，こうした喫煙者の配慮の結果，ETS曝露があったとしてもその絶対値が低く，PAHs曝露量には反映しなかった可能性もある。ETS曝露レベルと尿中1-OHP濃度の関連についてのより明確な検証を行うには，ETS曝露のバイオマーカー（たとえば尿中コチニンなど）との相関をみるといった検討の余地がある。

3. 土壌の寄与

調査対象である小児らは同じ幼稚園で過ごしており，また住居も同一地域であったため，接触のある土壌環境は類似していたと想定できる。この想定のもと，採尿前日に屋外で遊んだ時間または普段のマウジング行動の頻度が多いほど土壌経由でPAHsを多く摂取していると考え，その程度ごとにグループ分けして統計解析を行った (図3，表3)。しかしどちらも尿中1-OHP濃度との関連はみられなかった。また，より長期的な土壌接触頻度との関連も見いだすことはできなかった。以上より対象小児の土壌直接摂取は，PAHs曝露にほとんど寄与していなかったと考えられる。

ただし，この解析も土壌摂食量や土壌中PAHs濃度を直接測定したわけではないため，本研究の対象小児の土壌からのPAHs曝露量は定量的には明瞭ではない。例えばTsukataniら (22) は福岡県久留米市の土壌中B[a]P濃度を調査しており，一般道路沿いで最大86ng/gを報告している。この場合，小児の土壌摂取量が200mg/日 (23)，その他のB[a]P曝露量を成人の食事由来の曝露量92ng/日 (20) の7割と仮定すると，土壌の直接摂取にともなうB[a]P曝露量の寄与は，総曝露量の20%近くになると試算できる。また坂本ら (24) や竹田ら (25) は場所によってさらに高濃度のPAHsを観測している。たとえば坂本らは，神奈川県川崎市の幹線道路沿い土壌中に700ng/gのB[a]Pを観測したが (24)，この場合の土壌の寄与は総曝露量の70%に及ぶ可能性がある。このため特に幹線道路沿いなど土壌中PAHs濃度の高い可能性のある地域については，実測値に基づいた土壌からの曝露量評価を行なう必要がある。

4. 母子の尿中1-OHP濃度の差と関連

表1に示した結果より，小児の尿中1-OHP濃度は母親の約2倍であり，統計的に有意に高かった。これはHuangらの調査における成人-小児の関係とよく似た傾向であった。また母子の尿中1-OHP濃度には有意な正の相関もみられた (図4)。

この差の原因の一つの可能性として，母子の体重あたりの食事摂取量に2倍ほど差があることが要因と考えられる。厚生労働省，H15国民健康・栄養調査 (26) に基づき，小児と成人の平均摂取カロリー，体重から体重あた

りの摂取カロリー量を比較すると，小児は体重あたり76kcal，成人は34kcalであり，本研究で得られた尿中1-OHP濃度の小児・母親の関係とよくあっている。もし生体内でのPAHsあるいは1-OHP代謝に差がなく，体重当りのクレアチニン排泄量が等しければ，母子間の尿中1-OHP濃度の差異は体重当たりの食事摂取量（およびそれに伴うPAHs曝露量）によって説明できると考えられる。

これまでも指摘されてきて (9, 18, 20)，本研究でもETSや土壌からの寄与は少ない，と付け加えたように，現代日本における一般公衆のPAHsの主要な曝露源は食物と考えられているが，食事の内容によってPAHs含有量は大きく異なることが既往研究において報告されている (20)。1日を通して小児と共有する時間が最も多いのは母親であると考えられ，母子の食事内容は概ね類似していると想定すれば，母子の尿中1-OHP濃度の間に見られた有意な正の相関（図4）は説明がつく。ただし実際には昼食（小児は弁当）や，外食時のメニュー，間食において母子の食事内容に違いがある可能性も考えられ，それが図4に示した大きなバラツキの原因であると考えられる。

小児の年齢別尿中1-OHP濃度について弱い負の相関がみられたが，これについても成長に伴って体重あたりの食物摂取量が減っていくために体重あたりのPAHs曝露量が少なくなったという可能性が考えられる。しかしこの解釈が正当なものであるかどうか今後の検証が必要である。

V. 結　　論

本研究において，尿中1-OHP濃度から推定したPAHs曝露レベルは，近年の国内外の一般公衆についての報告と同レベルであった。また土壌摂食やETS曝露は小児のPAHs曝露に寄与しているという結果は得られなかった。大気からの寄与は少ないという既往研究結果を考えると，小児・成人ともに日本人におけるPAHs曝露は主に食事由来であるという可能性が考えられた。また成人と小児における尿中1-OHP濃度の差についても成人と小児の体重あたりの食物摂取量の違いによって説明できる可能性があった。これは一般公衆で非喫煙者の主なPAHs曝露源が食物である，という既往の国内外の知見と整合性をもつ推測ではあるが，今後はこの推測をさらに検証することで，日本人小児および成人のPAHs曝露を低減化するための対策立案に資するものと考えられる。

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Premature ovarian failure and androgen receptor gene CAG repeat lengths weighted by X chromosome inactivation patterns

The CAG repeat lengths weighted by X-inactivation ratios were significantly shorter in 58 Japanese patients with premature ovarian failure (POF) than in 42 age-matched control females with normal menses. The results suggest that short CAG repeats with a relatively high androgen receptor function may constitute a susceptibility factor for the development of POF. (Fertil Steril® 2009;91:649–52. ©2009 by American Society for Reproductive Medicine.)

Premature ovarian failure (POF) is a heterogeneous condition defined by the triad of primary or secondary amenorrhea, hypergonadotropism, and hypoestrinism in females less than 40 years old (1). While POF is frequently observed in females with sex chromosome aberrations, it also occurs in females with normal karyotypes (1). Although underlying factors for POF have been poorly elucidated in females with normal karyotypes, various genetic and environmental factors have been implicated in the development of POF. For example, mutations of several genes such as *BMP15*, *FOXL2*, and *NOBOX* as well as premutations of *FMR1* are known to cause POF (2–5), and several candidate genes such as *LHX8* and *GDF9* have been identified (6). Furthermore, chemotherapy, radiation, and autoimmune dysfunction also constitute risk factors for POF (1).

The androgen receptor (AR) plays a crucial role in sex development by mediating the biological effects of androgens (7). The *AR* gene resides on Xq12 and is made up of eight exons. Exon 1 harbors a highly polymorphic CAG repeat encoding a polyglutamine tract, and functional studies with different CAG repeat numbers have indicated an inverse relationship between the CAG repeat number and the transactivation function of *AR* (7). Consistent with this, the CAG repeat polymorphism is known to constitute a susceptibility factor for various androgen-related diseases in males (7). For example, while both positive and negative results have been reported, overall data from a large number of association studies argue that the CAG repeats tend to be long in males with undermasculinized genitalia and spermatogenic dysfunction and short in those with prostate cancers (7–9).

Similar association studies have also been performed in females with hirsutism and polycystic ovary syndrome (PCOS) together with X-inactivation analysis, revealing both positive and negative results (10–14). This would not necessarily be inconsistent with the CAG repeat polymorphism functioning as a susceptibility factor for androgen-related diseases in females as well as in males because the susceptibility effect may be detected in some patient groups but not in other patient groups. However, the data remain scanty, and further studies are necessary to draw a certain conclusion as to whether the CAG repeat polymorphism forms a susceptibility factor for androgen-related disorders in females. Thus, we performed CAG repeat length and X-inactivation analyses in POF patients because ovarian function is subject to androgen effects (1).

We studied 58 Japanese patients with POF. The menarcheal age ranged from 10 to 15 years (mean ± SD, 12.7 ± 1.2 years; menarcheal age in normal Japanese girls, 12.3 ± 1.3 years), and the age of POF onset (amenorrhea persisting ≥6 months) ranged from 13 to 39 years (median, 30 years). At the first medical examination, serum FSH was 44–245 IU/L (median, 94 IU/L), LH was 6–70 IU/L (median, 28 IU/L), and elevated FSH was repeatedly observed. Serum E_2 was undetectable in 45 patients and ranged from 10 to 72 pg/mL (35 to 250 pmol/L) in 13 patients. Serum T was not measured.

All 58 patients satisfied the following 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 the coding regions of *BMP15* and *GDF9*, and [6] no *FMR1* premutation. Two patients were familial cases with a similarly affected sister and/or mother, and the remaining 56 patients were sporadic cases. For controls, DNA samples from 42 Japanese females with proven fertility and normal menses aged 22–45 years (median, 34 years) were obtained from

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Reprint requests: Tsutomu Ogata, M.D., Department of Endocrinology and Metabolism, National Research Institute for Child Health and Development, Tokyo 157-8535, Japan (FAX: 81-3-5494-7026; E-mail: tomogata@nch.go.jp).

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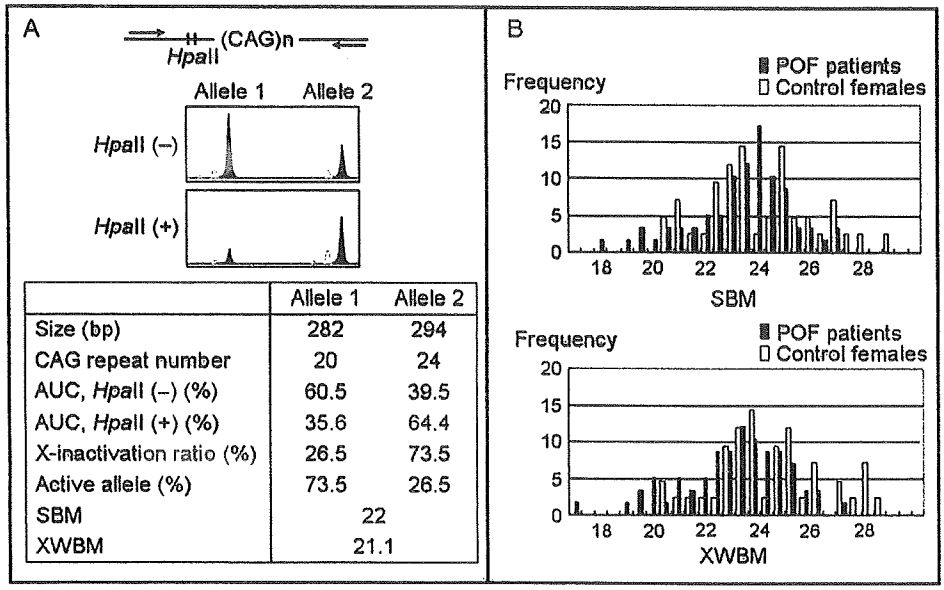
the Japanese Collection of Research Bioresources and similarly analyzed with permission. This study was approved by the Institutional Review Board committees of the investigators' affiliations. There is no conflict of interest.

CAG repeat length and X-inactivation analyses were performed by the previously reported method (15), with some modifications. In brief, leukocyte genomic DNA was polymerase chain reaction (PCR) amplified with a fluorescent labeled forward primer and an unlabeled reverse primer flanking the CAG repeat region and the two methylation sensitive *Hpa*II sites at exon 1 of *AR*, before and after *Hpa*II digestion (Fig. 1). The primer sequences and the PCR conditions were as described elsewhere (15). PCR products were obtained from both active and inactive X chromosomes before *Hpa*II digestion and from inactive X chromosomes alone after *Hpa*II digestion. For the CAG repeat length analysis, the PCR products obtained before *Hpa*II digestion were determined for size on an ABI PRISM 3100 autosequencer using GeneScan (Applied Biosystems, Norwalk, CT). Furthermore, to confirm the precise CAG repeat number, 12 PCR products of different sizes on GeneScan were subjected to direct sequencing on the autosequencer. For the X-inactivation analysis, the PCR products obtained before and after *Hpa*II digestion were examined for area under curve on the autosequencer. The X-inactivation ratio was calculated using the area under curve after compensation for unequal amplification of the two alleles caused by the difference in the product size. The CAG repeat number of each subject was obtained as the simple biallelic mean (SBM) and as the X-weighted biallelic mean (XWBM). The XWBM was calculated using the X-inactivation ratio and was expressed as a rounded number by increments of 0.5.

FIGURE 1

CAG repeat length and X-inactivation analyses. (**A**) Representative results. PCR amplification has been performed with a fluorescent labeled forward primer and an unlabeled reverse primer (arrows) flanking the CAG repeat region and the two methylation sensitive *Hpa*II sites at exon 1 of *AR*. Before *Hpa*II digestion, two alleles have been delineated on the autosequencer; allele 1 is 282 bp long and contains 20 CAG repeats, and allele 2 is 294 bp long and contains 24 CAG repeats. The difference in the area under curve (AUC) between the two alleles is primarily due to the short allele being more easily amplified than the long allele. The small 279 and 291 bp peaks are by-products caused by the slippage phenomenon. After *Hpa*II digestion, the two alleles have been detected, and the difference in the AUC pattern before and after the *Hpa*II digestion is primarily caused by noneven X-inactivation. The X-inactivation ratio, which is a mirror image of the active allele ratio, is calculated using the AUCs before and after *Hpa*II digestion. In this patient, the allele 2 is more preferentially inactivated than the allele 1, and the allele 1 and the allele 2 are expressed in 73.5% and 26.5% of leukocytes, respectively. Thus, the SBM is obtained as 22, and the XWBM is calculated as 21.1. (**B**) Distribution of the SBMs and the XWBMs in patients with POF and control females. The XWBM has been obtained as a rounded number by increments of 0.5; for example, calculated XWBM values from 22.75 to 23.24 have been rounded as 23, and those from 23.25 to 23.74 have been rounded as 23.5.



	Allele 1	Allele 2
Size (bp)	282	294
CAG repeat number	20	24
AUC, *Hpa*II (–) (%)	60.5	39.5
AUC, *Hpa*II (+) (%)	35.6	64.4
X-inactivation ratio (%)	26.5	73.5
Active allele (%)	73.5	26.5
SBM	22	
XWBM	21.1	

Sugawa. POF and AR CAG repeat polymorphism. Fertil Steril 2009.

Representative results and the distributions of the SBMs and the XWBMs are shown in Figure 1. The SBMs and the XWBMs were found to follow the normal distribution in both the POF patients and the control females by the χ^2-test, and the variances were shown to be similar between the two groups by the *F*-test. Thus, the Student's *t*-test was employed for the statistical analysis, showing that the SBMs were comparable between the POF patients and the control females (mean ± SD, 23.3 ± 2.0 vs. 24.1 ± 2.1; $P=.07$), whereas the XWBMs were mildly but significantly shorter in the POF patients than in the control females (mean ± SD, 23.2 ± 2.1 vs. 24.2 ± 2.2; $P=.02$). Neither the SBM nor the XWBM was found to be correlated with the menarcheal age ($r = -0.02$; $P=.90$), the age of POF onset ($r = 0.08$, $P=.58$), the serum FSH value ($r = 0.01$, $P=.94$), and the LH value ($r = -0.05$, $P=.78$) by the Spearman's ρ test.

The XWBM was mildly but significantly shorter in the patients with POF than in the control females, although the SBM was comparable between the two groups of subjects. In this context, while the AR function has not been compared between the two groups of subjects in this study, the previous studies have indicated an inverse relationship between the CAG repeat number and the AR function (7). Thus, a relatively high AR function in somatic cells may be a susceptibility factor for the development of POF because the AR function in somatic cells would be better reflected by the XWBM than by the SBM. Since *AR* is clearly expressed in the granulosa cells of developing follicles (16), increased AR function may affect the follicular cell function, facilitating the development of POF. Indeed, androgen excess in several conditions such as 21-hydroxylase deficiency and PCOS is known to impair ovarian function (1, 17), although there has been no report documenting the relationship between androgen excess and POF. One may argue that POF can also result from dysfunction of oocytes in which the AR function would simply be reflected by the SBM rather than the XWBM because the two X chromosomes remain active in oocytes (18). However, the relevance of an oocyte factor to POF is unlikely in terms of the AR function because *AR* is not expressed in oocytes (16).

The SBM and the XWBM were not correlated with the menarcheal and POF onset ages or the serum gonadotropin values. This would at least in part be due to variations in genetic and environmental factors influencing menarcheal and menopausal ages and hormonal values.

Several points should be made with respect to the present study. First, most of the control females were less than 40 years of age. This may have affected the results of this study because some of them may develop POF at a later age. Second, the X-inactivation pattern was examined for leukocytes in this study as well as in the previous studies of the CAG repeat polymorphism in females (10–14). Thus, although the X-inactivation ratio is similar among different tissues in most individuals (19), the XWBM may more or less be different between leukocytes and target tissues such as ovarian cells. Third, it remains to be examined whether CAG repeats tend to be short in other POF patients as well. Furthermore, POF may actually be associated with long CAG repeats with a relatively low AR function in ovarian follicular cells because POF is exhibited by female mice lacking *AR* (20). Thus, further studies are obviously necessary to examine the notion that short CAG repeats constitute a susceptibility factor for the development of POF.

Fumihiro Sugawa, B.Sc.[a,b]
Yuka Wada, M.D.[a]
Tetsuo Maruyama, M.D.[c]
Hiroshi Uchida, M.D.[c]
Bunpei Ishizuka, M.D.[d]
Tsutomu Ogata, M.D.[a,b]
[a] *Department of Endocrinology and Metabolism, National Research Institute for Child Health and Development,* [b] *Biomedical Science PhD Program, Tokyo Medical and Dental University, and* [c] *Department of Obstetrics and Gynecology, Keio University School of Medicine, Tokyo, and* [d] *Department of Obstetrics and Gynecology, St. Marianna University School of Medicine, Kawasaki, Japan*

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Short Report

CHD7 mutations in patients initially diagnosed with Kallmann syndrome – the clinical overlap with CHARGE syndrome

Jongmans MCJ, van Ravenswaaij-Arts CMA, Pitteloud N, Ogata T, Sato N, Claahsen-van der Grinten HL, van der Donk K, Seminara S, Bergman JEH, Brunner HG, Crowley Jr WF, Hoefsloot LH. *CHD7* mutations in patients initially diagnosed with Kallmann syndrome – the clinical overlap with CHARGE syndrome.
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Kallmann syndrome (KS) is the combination of hypogonadotropic hypogonadism and anosmia or hyposmia, two features that are also frequently present in CHARGE syndrome. CHARGE syndrome is caused by mutations in the *CHD7* gene. We performed analysis of *CHD7* in 36 patients with KS and 20 patients with normosmic idiopathic hypogonadotropic hypogonadism (nIHH) in whom mutations in *KAL1*, *FGFR1*, *PROK2* and *PROKR2* genes were excluded. Three of 56 KS/nIHH patients had *de novo* mutations in *CHD7*. In retrospect, these three *CHD7*-positive patients showed additional features that are seen in CHARGE syndrome. *CHD7* mutations can be present in KS patients who have additional features that are part of the CHARGE syndrome phenotype. We did not find mutations in patients with isolated KS. These findings imply that patients diagnosed with hypogonadotropic hypogonadism and anosmia should be screened for clinical features consistent with CHARGE syndrome. If such features are present, particularly deafness, dysmorphic ears and/or hypoplasia or aplasia of the semicircular canals, *CHD7* sequencing is recommended.

MCJ Jongmans[a], CMA van Ravenswaaij-Arts[b], N Pitteloud[c], T Ogata[d], N Sato[d], HL Claahsen-van der Grinten[e], K van der Donk[a], S Seminara[c], JEH Bergman[b], HG Brunner[a], WF Crowley Jr[c] and LH Hoefsloot[a]

[a]Department of Human Genetics, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands, [b]Department of Genetics, University Medical Center Groningen, Groningen University, The Netherlands, [c]Harvard Reproductive Endocrine Sciences Center and The Reproductive Endocrine Unit, Department of Medicine, Massachusetts General Hospital, Boston, MA, USA, [d]Department of Endocrinology and Metabolism, National Research Institute for Child Health and Development, Tokyo, Japan, and [e]Department of Pediatric Endocrinology, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands

Key words: anosmia – CHARGE syndrome – *CHD7* gene – hypogonadotropic hypogonadism – Kallmann syndrome

Corresponding author: Marjolijn CJ Jongmans, MD, Department of Human Genetics, Radboud University Nijmegen Medical Centre, PO Box 9101, 6500 HB Nijmegen, The Netherlands.
Tel.: 31-24-3613946;
fax: 31-24-3668774;
e-mail: m.jongmans@antrg.umcn.nl

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Kallmann syndrome (KS) is a congenital disorder that combines hypogonadotropic hypogonadism and anosmia (1). Three modes of inheritance have been described: X-linked recessive, autosomal dominant and more rarely autosomal recessive. To date, several genes have been identified to cause KS, either alone or in combination. Mutations in these genes together account for approximately 30% of all cases. *KAL1* encodes the protein anosmin and is involved in the X-linked

form of KS (KAL1, OMIM #308700) (2, 3). Loss-of-function mutations in the fibroblast growth factor receptor-1 gene (*FGFR1*) cause a form of KS (KAL2, OMIM #147950) that is generally inherited in an autosomal dominant way (4, 5). Dodé et al. reported in a further 10% of patients mutations in the prokineticin receptor-2 (*PROKR2*, KAL3, OMIM #607123) and prokineticin-2 (*PROK2*, KAL4, OMIM #607002) genes, encoding a cell surface receptor and one of its ligands, respectively (6). Mutations of the ligand, *PROK2*, can cause KS as well as normosmic idiopathic hypogonadotropic hypogonadism (nIHH) within the same family (6, 7). The same intrafamilial phenotypic variability is seen in patients with *FGFR1* mutations (4). Thus, KS is a phenotypically and genotypically heterogeneous disorder. Not only the degree of hypogonadism and anosmia may vary significantly but also other symptoms including bimanual synkinesia and dental agenesis (*KAL1* and *FGFR1*), renal anomalies (*KAL1*) and cleft lip/palate (*FGFR1*) occur with variable frequency (8).

CHARGE syndrome (OMIM #214800) is an autosomal dominant condition characterized by a variety of congenital anomalies including coloboma, heart defects, choanal atresia, retarded growth and development, genital hypoplasia, ear anomalies and deafness. Other commonly observed congenital defects are semicircular canal hypoplasia, facial nerve palsy, cleft lip/palate and tracheo-esophageal fistula (9). Our group has discovered *CHD7* as the causative gene in CHARGE syndrome (10). Since this discovery, several authors have reported on the phenotypic spectrum of *CHD7*-positive patients, including patients without typical CHARGE syndrome (11–13). Therefore, we presume that the mild end of the phenotypic spectrum of *CHD7* mutations is not yet completely explored.

Recent studies revealed that anosmia and abnormal olfactory bulb development, as well as hypogonadotropic hypogonadism, are almost consistent findings in CHARGE syndrome, indicating that the key features of KS are also present in CHARGE syndrome (14–16). For this reason, it has been suggested by others that *CHD7* may be considered a candidate locus in suspected KS cases without known mutations (8). This hypothesis is worthwhile exploring, also because mutations in *CHD7* can result in a much milder phenotype than the classical CHARGE syndrome phenotype. Therefore, we sequenced *CHD7* in a large group of patients diagnosed as KS or nIHH but without mutations in *KAL1*, *FGFR1*, *PROK2* and *PROKR2*.

Materials and methods

Patients

A cohort of seven Japanese patients with a clinical diagnosis of KS, without mutations in *KAL1*, *FGFR1*, *PROK2* and *PROKR2*, was screened for *CHD7* mutations (17). The diagnosis KS in this cohort was based on an underdevelopment of secondary sexual characteristics in combination with anosmia or hyposmia. Subsequently, the cohort was enlarged by 49 *KAL1*, *FGFR1*, *PROK2* and *PROKR2* negative North American patients with KS or nIHH. GnRH deficiency in this cohort was defined by (a) absent/incomplete puberty by age 18 year; (b) serum testosterone <100 ng/dl in men or estradiol <20 pg/ml in women in association with low or normal levels of serum gonadotropins; (c) otherwise normal pituitary function; (d) normal serum ferritin concentrations; and (e) normal magnetic resonance imaging (MRI) of the hypothalamic-pituitary region (5).

The patients in whom *CHD7* mutations were identified were carefully evaluated for clinical features of CHARGE syndrome. The *CHD7* gene was analyzed in the parents. The patients or their legal representatives gave informed consent for the DNA studies and the collection of clinical data. The studies were approved by the institutional review boards.

Mutation screening

DNA was isolated according to standard procedures. The 37 coding exons of the *CHD7* gene (exon 2–38, accession number NM_017780, NCBI) and their flanking intron sequences were amplified by polymerase chain reaction (PCR). Subsequently, sequence analysis was performed using a 3730 automated sequencer (Applied Biosystems, Foster City, CA). Primer information and PCR conditions are given in a previous report of our group (11).

The DNA samples of 11 mutation-negative patients were subsequently screened for exon deletions and/or duplications of the *CHD7* gene by multiplex ligation probe dependent amplification (MLPA) analysis (Table 1). We used a commercially available set of probes, the SALSA P201 kit (MRC-Holland, Amsterdam, The Netherlands; http://www.mrc-holland.com). Further details are described in our recent report on MLPA analysis of the *CHD7* gene (18).

Results

The *CHD7* gene was first screened in a cohort of seven *KAL1*, *FGFR1*, *PROK2* and *PROKR2*

Table 1. Clinical characteristics of all patients and results of *CHD7* analysis[a]

No.	Sex	Diagnosis	Additional features	Family	Mutation *CHD7*	Parents	MLPA performed
1	M	KS	Dental agenesis, high-arched palate, unilateral perceptive deafness and short stature	Sp	c.8803G>T; p.Glu2935X; exon 38	*De novo*	–
2	M	KS	Cleft palate, auricular dysplasia, nystagmus, bilateral perceptive deafness and hypoplasia of semicircular canals	Sp	c.6347T>A; p.Ile2116Asn; exon 31	*De novo*	–
3	F	KS		Sp	–		–
4	F	KS		Sp	–		–
5	M	KS	High-arched palate	Sp	–		–
6	M	KS	Ptosis	Sp	–		–
7	M	KS		Sp	–		–
8	F	KS	Facial nerve palsy, bilateral colobomas, cleft lip/palate, deafness, short stature and developmental delay	Sp	c.6070C>T; p.Arg2935X; exon 30	*De novo*	–
9	F	KS		Fam	–		+
10	M	KS		Fam	–		–
11	F	KS	Crohn's disease, syndactyly	Fam	–		–
12	M	KS		Fam	–		–
13	M	KS		Sp	–		+
14	F	KS		Sp	–		–
15	F	KS,	Choanal atresia	Fam	–		+
16	M	KS		Fam	–		–
17	M	KS	Congenital deafness and Hirschsprung's disease	Sp	–		–
18	F	KS		Fam	–		–
19	M	KS		Fam	–		–
20	F	KS		Fam	–		+
21	F	KS	Hearing impairment	Fam	–		–
22	M	KS	Deafness	Sp	–		–
23	F	KS	Multiple cranial nerve abnormalities	Sp	–		+
24	F	KS		Fam	–		–
25	F	KS		Sp	–		+
26	M	KS		Fam	–		–
27	M	KS	Hearing impairment	Sp	–		–
28	M	KS		Fam	–		–
29	M	KS		Fam	–		–
30	M	KS	Cryptorchidism	Fam	–		–
31	M	KS		Fam	–		–
32	F	KS	Narrow palate	Fam	–		–
33	F	KS	High-arched palate and hyperlaxity of hand joints	Fam	–		–
34	M	KS	Macrocephaly, hypertelorism, high-arched palate, ataxia, Dandy Walker malformation and developmental delay	U	–		–
35	M	KS		Fam	–		–
36	M	Partial KS	Spinal muscular atrophy	Sp	–		–
37	M	IHH, KS in family	Cardiac septum defect	Fam	–		+
38	M	IHH, KS in family	Hearing impairment	Fam	–		+
39	M	IHH, KS in family		Fam	–		–
40	F	IHH		Fam	–		+
41	M	IHH		Sp	–		+
42	F	IHH	Cardiac septum defect	Sp	–		+
43	M	IHH	Cryptorchidism	Fam	–		–
44	M	IHH	Growth hormone deficient	Fam	–		–
45	F	IHH		Fam	–		–
46	F	IHH		Fam	–		–
47	M	IHH	Cryptorchidism, blind, seizures, mental retardation and short stature	Sp	–		–
48	F	IHH		Fam	–		–
49	M	IHH	Ataxia	Sp	–		–
50	F	IHH		Fam	–		–
51	F	IHH		Fam	–		–

Table 1. Continued

No.	Sex	Diagnosis	Additional features	Family	Mutation *CHD7*	Parents	MLPA performed
52	M	IHH		Fam	–		–
53	M	IHH		Fam	–		–
54	M	IHH	Developmental delay and high-arched palate	Fam	–		–
55	M	IHH		Fam	–		–
56	F	IHH		Fam	–		–

F, female; Fam, familial; IHH, idiopathic hypogonadotropic hypogonadism; KS, Kallmann syndrome (IHH + anosmia); M, male; MLPA, multiplex ligation probe amplification; partial KS, patient with IHH and anosmia, with some degree of spontaneous pubertal development; Sp, sporadic; U, unknown.
[a]Patients 1–7 are of Japanese descent and patients 8–56 are from North America.

negative patients of Japanese descent (five males, two females). All had hypogonadotropic hypogonadism and anosmia, whereas some had additional symptoms. Their clinical features are summarized in Table 1, and patient 2 is shown in Fig. 1.

In two of the seven patients, a heterozygous mutation in *CHD7* was identified: one nonsense mutation (c.8803G>T; p.Glu2935X) and one missense mutation (c.6347T>A; p.Ile2116Asn). The mutations were proven to be *de novo* in both patients and were not present in 600 alleles of healthy controls.

The study cohort was extended by 49 North American patients (28 males, 21 females), including 29 patients with KS and 20 with nIHH of whom three had a positive family history for KS. Some of these patients had additional phenotypic features (Table 1). In one of the patients (patient 8), a *de novo* pathogenic nonsense mutation in *CHD7* was found (c.6070C>T; p.Arg2935X).

Fig. 1. Lateral view of patient 2. Note the dysmorphic ears with absence of the earlobe and the lower helical fold, and a triangular concha. These dysmorphism are typical for CHARGE syndrome.

As whole exon deletions or duplications will be missed by sequence analysis, we performed MLPA analysis. Due to a limited amount of available DNA, we were only able to finish this analysis in 11 patients. Two patients with a relatively high suspicion for CHARGE syndrome based on the features choanal atresia and multiple cranial nerve anomalies (respectively, patient 15 and 23; Table 1) were among those 11 patients. No exon copy number alterations were found.

The main features of the three patients carrying a mutation in *CHD7* are given in Table 1. All three patients were proven to be anosmic by formal smell tests. Audiometry revealed a left-sided hearing impairment of 70 dB in patient 1, a bilateral hearing impairment of 60–90 dB in patient 2 and left-sided complete sensorineural deafness and right-sided partial conductive hearing impairment in patient 8. Patient 1 had agenesis of four permanent teeth, the first upper and lower molars. No choanal atresia or heart defects were present in patients 1, 2 and 8. Colobomas were present in patient 8 but excluded by fundoscopy in patients 1 and 2. Patient 2 experienced feeding difficulties during infancy, but these were ascribed to the cleft palate. The dysmorphisms of the ears of patient 2 are very characteristic for CHARGE syndrome with absence of the earlobe and the lower helical fold, and a typical triangular concha (Fig. 1). After identification of the *CHD7* mutation, a CT scan of the os petrosum showed bilateral hypoplasia of the semicircular canals. In patients 1 and 8, imaging studies of the temporal bones were not possible. Upon re-evaluation, patient 8 has not only deafness and bilateral colobomas but also left-sided facial nerve palsy, cleft lip and palate, short stature and developmental delay.

In retrospect, patients 2 and 8 have typical CHARGE syndrome according to the commonly used clinical criteria (9), while patient 1 has only some features of this syndrome.

Discussion

Hypogonadotropic hypogonadism is a frequent feature in CHARGE syndrome. Chalouhi et al. tested the olfactory function of 14 children with CHARGE syndrome and showed that all children had some degree of olfactory deficiency (14). Pinto et al. showed that olfactory deficiency and abnormal olfactory bulbs were present in all 18 CHARGE syndrome patients in their cohort (15).

These observations prompted us to analyze the *CHD7* gene in 36 patients with KS and 20 patients with nIHH lacking mutations in *KAL1*, *FGFR1*, *PROK2* and *PROKR2*. *CHD7* mutations were identified by sequence analysis in 2 of 7 Japanese KS patients and in 1 of 49 KS/nIHH North American patients. By routine sequencing of the *CHD7* gene, we may have missed mutations located deep in introns or in the promoter region. Furthermore, MLPA analysis could not be performed in all patients.

Hypogonadism in KS is caused by GnRH deficiency. GnRH neurons of the forebrain are thought to originate from the nasal placode. During embryonic development, they migrate alongside the olfactory axons toward the hypothalamus. Mutations in *KAL1*, *FGFR1*, *PROKR2* and *PROK2* can result in hypogonadotropic hypogonadism and anosmia. Therefore, the protein products of these genes are thought to be involved in this combined migration process (8, 19). Because hypogonadotropic hypogonadism and anosmia are frequently present in CHARGE syndrome as well, it is possible that the same embryonic migration process is disturbed in CHARGE syndrome. *CHD7* encodes a protein of the chromodomain (chromatin organization modifier) family. This family shares a unique combination of functional domains consisting of two N-terminal chromodomains, followed by a SWI2/SNF2-like ATPase/-helicase domain and a DNA-binding domain. It is assumed that *CHD* protein complexes affect chromatin structure and gene expression and thereby play important roles in regulating embryonic development (20). Therefore, one might speculate that *CHD7* has a possible influence on the expression or actions of *KAL1*, *FGFR1*, *PROK2* and/or *PROKR2* during development. However, because mutations in these genes account for only 30% of all KS cases, it is possible that *CHD7* impacts on other yet undiscovered, KS genes.

We identified a *de novo CHD7* mutation in three patients initially diagnosed with KS. The two nonsense mutations are known to be pathogenic. The missense mutation p.Ile2116Asn is not located in one of the known protein domains of the CHD7 protein, but it concerns a drastic amino acid change that has not been detected in over 600 control alleles. Furthermore, the p.Gly2108Arg mutation has been shown to be associated with CHARGE syndrome in two families with a variable phenotype, indicating that this part of the protein probably has an important function (12). This indicates that the p.Ile2116Asn mutation is possibly pathogenic.

In retrospect, two of the three *CHD7*-positive patients (patients 2 and 8) had typical CHARGE syndrome with the presence of at least three major features (9). Patient 1 presented with only two additional CHARGE features (short stature and unilateral hearing impairment), although one should notice that vestibular function was not tested in this patient.

From this study, we conclude that it is important to evaluate patients with hypogonadotropic hypogonadism and anosmia for clinical features characteristic of CHARGE syndrome. All three patients were proven to be anosmic. Therefore, the chance to find a *CHD7* mutation seems higher in anosmic patients although the study group is too small to conclude that *CHD7* mutations cannot occur in patients with normosmic IHH. Indeed some patients with CHARGE syndrome are able to smell (personal observations). Because all three patients suffered from hearing impairment, it is tempting to regard this feature as discriminating. However, sensorineural hearing impairment is also an associated feature in males with *KAL1* mutations. Thus, hearing abnormalities may be a sensitive but not very specific symptom of *CHD7* mutations. Hypoplasia or aplasia of the semicircular canals is a much more consistent feature in CHARGE syndrome, even in mildly affected patients (9, 12). Therefore, history taking regarding balance disturbances and gross motor development might reveal indicative information for the presence of a *CHD7* mutation. Abadie et al. have described a specific pattern of postural behavior related to vestibular anomalies in CHARGE syndrome. They noticed a frequent inability to crawl on all fours without resting the head on the floor (5-point crawl), a prolonged duration of standing with support stage and an inability to ride a bike without stabilizers (21). After the first years of life, balance disturbances may not be unequivocally present as a result of visual compensation. In these patients, disequilibrium in the dark is a helpful indication of vestibular deficit. If there is doubt about the vestibular function, screening for vestibular areflexia or imaging of the semicircular canals will be helpful. In the newborn, agenesis of the semicircular canals can be visualized on plain profile X-ray of the

skull (9). In older patients, computerized tomography or MRI is necessary.

Finally, dilated fundus examination can be performed to reveal an optic disc coloboma. A less invasive, but of course also less accurate method, would be to ask for the presence of an optic field defect.

CHD7 screening in the large North American cohort revealed only one mutation. In general, these patients underwent a more extensive clinical work-up (5). From this cohort, we learned that it is not useful to screen the *CHD7* gene in each patient diagnosed with KS or nIHH; additional CHARGE features should be present. Such additional features do not imply that a *CHD7* mutation will be present as has been demonstrated by patient 15 who has choanal atresia but no *CHD7* mutation.

The patients carrying a mutation in *CHD7* in this cohort and the mild *CHD7*-positive patients reported by us in a previous study (12) show that the current diagnostic criteria cannot always discriminate between patients with and without a mutation in *CHD7* (9, 12).

We conclude that it is useful to screen patients with hypogonadotropic hypogonadism and anosmia for clinical features consistent with CHARGE syndrome, particularly hearing impairment, vestibular dysfunction and dysmorphisms of the ears. If additional features of CHARGE syndrome are present, *CHD7* sequencing is recommended.

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Informed consent

All patients or their legal representatives gave informed consent for the DNA studies and the collection of clinical data. The studies were approved by the institutional review boards. Additional informed consent for publication was obtained of the patient represented by his photograph in this manuscript.

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