Table 2
Clinical characteristics of 48 patients with Type 2 diabetes mellitus: incidence of retinopathy, peripheral neuropathy and hypertension

Group	Retinopathy			Peripheral neuropathy		Hypertension	
	Absent	Simple	Pre-proliferative + proliferative	Absent	Present	Absent	Present
Ia	11	0	2	5	8	8	5
Ib	3	0	1	3	1	3	1
IIa	8	1	2	7	4	8	3
IIb	2	1	3	4	2	2	4
IIc	1	0	7	3	5	1	7
III	1	0	5	1	5	1	5
Total	26	2	20	23	25	23	25

Number of patients in each group is shown. Simple, simple diabetic retinopathy; pre-proliferative, pre-proliferative diabetic retinopathy; proliferative, proliferative diabetic retinopathy.

The patients on anti-hypertensive drugs, or with systolic blood pressure >160 mmHg, or diastolic blood pressure >95 mmHg were considered to have hypertension (25 out of 48 Type 2 diabetic patients) (Table 2).

2.2. Radioimmunoassay

Plasma IR-UII concentrations were measured by radioimmunoassay following the extraction using Sep-Pak C_{18} cartridges (Waters Associates, Milford, MA, USA), as previously reported [20,21]. Urine samples were extracted with Sep-Pak C_{18} cartridges. Three ml urine was acidified with 6 ml of 0.75 mol/l acetic acid and loaded onto the cartridge, which was pretreated with 10 ml acetonitrile, 10 ml methanol and then 10 ml of 0.75 mol/l acetic acid. After washing the cartridge with 10 ml of 0.75 mol/l acetic acid, the peptide was eluted from the cartridge with 2 ml of 60% (v/v) acetonitrile containing 0.1% trifluoroacetic acid. The eluate was air-dried, reconstituted with assay buffer and assayed as previously reported [20,21]. The recovery, which was determined by adding UII to the urine prior to the extraction, was more than 96% ($n = 5$). The cross reaction was about 1% with urotensin II-related peptide [16] (Peptide Institute, Osaka, Japan), but less than 0.001% with other peptides including endothelin-1, neuropeptide Y, urocortin 1, and somatostatin [19].

2.3. Chromatography

Chromatographic characterization of IR-UII in the plasma and urine was performed by reverse phase high performance liquid chromatography (HPLC) using a μBondapak C18 column (3.9 mm × 300 mm, Waters). The pooled plasma and urine obtained from normal subjects were extracted by Sep-Pak C18 cartridges and reconstituted in 0.1% (v/v) trifluoroacetic acid (TFA). The HPLC analysis was performed with a linear gradient of acetonitrile containing 0.1% TFA from 10% to 60% at a flow rate of 1 ml/min per fraction over 50 min. Each fraction (1 ml) was collected, dried by the air, reconstituted with assay buffer and assayed.

2.4. Statistics

Data are shown as mean ± S.E.M. unless otherwise stated. The statistical analysis was performed by one-way analysis of variance and Scheffe's multiple comparison test. Correlation was examined with Pearson's correlation coefficient.

3. Results

3.1. IR-UII in plasma and urine of patients with Type 2 diabetes mellitus: relationship with renal function

IR-UII was detectable in all plasma and urine samples. IR-UII concentrations were distributed from 2.5 to 24.0 fmol/ml in plasma and from 4.5 to 63.6 fmol/ml in urine. In 48 patients with Type 2 diabetes mellitus, plasma IR-UII concentrations showed a significant positive correlation with urinary IR-UII excretion ($r = 0.385$, $P = 0.0065$) and a significant negative correlation with IR-UII clearance ($r = -0.378$, $P = 0.0075$), but no significant correlation with fractional excretion of IR-UII (FE-UII) ($P = 0.937$). The plasma IR-UII concentrations, urinary IR-UII excretion and IR-UII clearance in control subjects and Type 2 diabetic patients are shown in Fig. 1 and fractional excretion of IR-UII (FE-UII) in Type 2 diabetic patients is in Fig. 2.

Plasma IR-UII concentrations were significantly elevated in patients with Type 2 diabetes mellitus when compared with control subjects (5.2 ± 0.4 fmol/ml, $P < 0.005$) ($F(3,54) = 10.618$, $P < 0.0001$). Plasma IR-UII levels were much higher in group III with Ccr < 30 ml/min (15.9 ± 2.2 fmol/ml) than in groups I and II (group I, 10.9 ± 0.9 fmol/ml; and group II, 10.8 ± 0.8 fmol/ml, $P < 0.05$) (Fig. 1A).

Urinary IR-UII excretion was significantly increased in group III (52.4 ± 14.8 pmol/day) by more than 1.8-fold compared with control subjects, groups I and II ($P < 0.005$) ($F(3,54) = 6.431$, $P < 0.001$) (Fig. 1B). There was no significant difference in urinary IR-UII excretion among these three groups (Fig. 1B). There was no significant difference in IR-UII clearance among the control subjects and three groups of Type 2 diabetic patients ($F(3,54) = 3.005$, $P > 0.05$) (Fig. 1C).

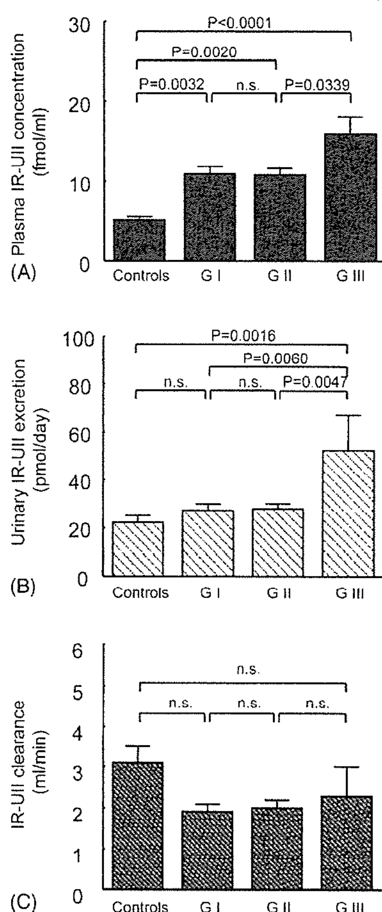

Fig. 1. (A) Plasma immunoreactive-urotensin II (UII) concentrations, (B) urinary excretion of immunoreactive-UII and (C) immunoreactive-UII clearance in 48 patients with Type 2 diabetes mellitus and 10 control subjects. The Type 2 diabetic patients were divided into three groups (group I, GI; group II, G2; and group III, GIII) according to their renal function (Table 1). Data are shown as the mean ± S.E.M.

FE-UII significantly increased as renal function decreases ($F(2,45) = 23.428$, $P < 0.0001$) (Fig. 2). Group III showed the highest FE-UII (0.13 ± 0.04 %), more than three-fold compared with groups I and II ($P < 0.0001$). The presence of microalbuminuria or proteinuria had a negligible effect on the

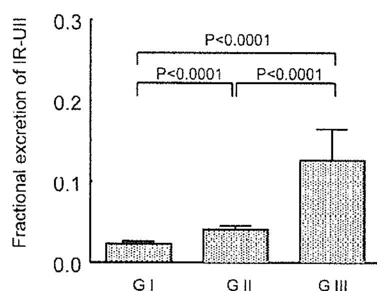

Fig. 2. Fractional excretion of immunoreactive-urotensin II (UII) in 48 patients with Type 2 diabetes mellitus. group I, GI; group II, GII; and group III, GIII. Data are shown as the mean ± S.E.M.

plasma IR-UII concentration or urinary IR-UII excretion in patients with Ccr ≥ 30 ml/min (groups I and II) ($P > 0.1$)(data not shown).

3.2. Relationship with other diabetic complications and clinical parameters

The presence of pre-proliferative or proliferative diabetic retinopathy had a negligible effect on the plasma IR-UII concentration ($F(2,45) = 0.745$, $P > 0.4$) or urinary IR-UII excretion ($F(2,45) = 1.644$, $P > 0.2$) in Type 2 diabetic patients. Fractional excretion of UII in patients with pre-proliferative or proliferative diabetic retinopathy, however, was significantly higher than those in patients without diabetic retinopathy (0.066 ± 0.015 and 0.031 ± 0.004%, respectively) ($F(2,45) = 3.468$, $P < 0.05$), which may reflect the accompanying renal dysfunction in these patients.

There were no significant differences in plasma IR-UII concentrations ($P = 0.0738$) or urinary IR-UII excretion ($P > 0.8$) between patients with and without diabetic peripheral neuropathy. The presence of hypertension had a negligible effect on the plasma IR-UII concentration or urinary IR-UII excretion in patients with Ccr ≥ 30 ml/min (groups I and II) ($P > 0.3830$).

We then examinated correlation of plasma IR-UII concentrations, urinary IR-UII excretion, IR-UII clearance and FE-UII to other clinical parameters in 48 patients with Type 2 diabetes mellitus. Creatinine clearance (Ccr) had significant negative correlations to urinary IR-UII excretion ($r = -0.336$, $P = 0.0191$) and FE-UII ($r = -0.625$, $P < 0.0001$). No significant correlation, however, was observed between plasma IR-UII concentrations and Ccr ($P = 0.1293$), fasting blood sugar level (FBS), hemoglobin A1c, body mass index (BMI), cardiothoracic ratio (CTR) on chest rentogenogram, urinary excretion of *N*-acetyl-glucosaminidase, ages or duration of diabetes mellitus in the 48 Type 2 diabetic patients ($P > 0.05$). There was also no significant relation between urinary excretion of IR-UII and FBS, hemoglobin Alc, BMI, CTR, urinary excretion of *N*-acetyl-glucosaminidase or duration of diabetes mellitus in these patients ($P > 0.05$). No significant relation was found again when these correlations were examined within each group with similar renal functions (groups I, II and III).

3.3. Chromatography

HPLC analysis of pooled normal plasma showed three immunoreactive peaks, one of which was eluted in the position of synthetic human UII (Fig. 3A). Urinary IR-UII consisted of at least six components; one of them was eluted in the position of synthetic human UII and two of them were eluted in the similar positions of immunoreactive peaks shown in the plasma (Fig. 3B).

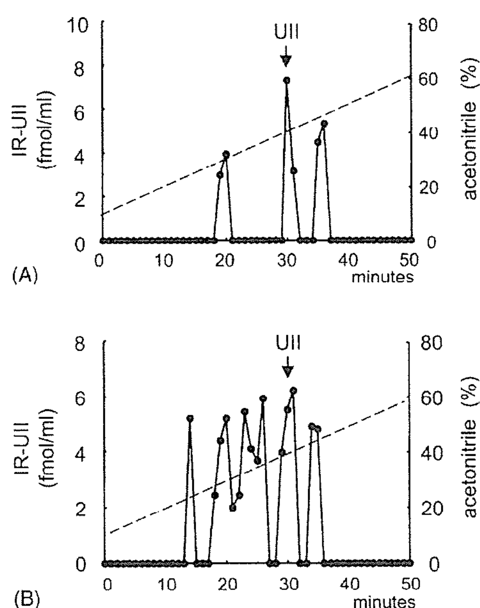

Fig. 3. Reverse phase high performance liquid chromatography of (A) the pooled plasma and (B) the urine extracts obtained from normal subjects. An arrow (UII) indicates the elution position of synthetic human urotensin II. A dotted line indicates a gradient of acetonitrile.

4. Discussion

The present study has shown high plasma IR-UII levels in Type 2 diabetic patients (Fig. 1A). The finding is compatible with our previous report on high plasma IR-UII levels in diabetic patients without renal failure [21]. In addition to diabetes mellitus and renal failure, heart failure [5,8,9] and liver cirrhosis [6] have been reported as the diseases having the elevated plasma IR-UII levels. Though increased production of UII in these pathological states were supposed, the precise mechanism of the elevation has not been clarified yet. The present study also has shown that Type 2 diabetic patients with advanced renal dysfunction had higher plasma IR-UII levels than those with normal or moderately decreased renal function. These results suggest that pathological state of Type 2 diabetes mellitus itself is a factor to elevate plasma IR-UII levels, and accompanying renal failure is another independent factor for the increased plasma IR-UII levels in Type 2 diabetic patients.

Two possible explanations are supposed for the elevated plasma IR-UII levels in the renal dysfunction: an increase of UII production in various organs, and a decrease of IR-UII excretion to urine. We therefore examined whether urinary excretion of IR-UII was decreased in diabetic patients with the renal dysfunction. Contrary to our expectation, diabetic patients with advanced renal dysfunction had increased urinary IR-UII excretion compared to those with normal to moderately decreased renal function. Increased urinary excretion of IR-UII was accompanied by increased levels of the fractional excretion of IR-UII in these patients. We previously reported increases in urinary excretion and fractional excretion of brain natriuretic peptide in patients with chronic renal failure [22]. Matsushita et al. reported that urinary IR-UII concentrations per gram creatinine were significantly increased in patients with renal tubular abnormality, but not with glomerular diseases, when compared with normal individuals [7]. Possible explanation for increased urinary excretion of IR-UII in group III may be an increased glomerular UII filtration due to the elevated plasma IR-UII levels and a decreased degradation of UII derived from plasma by the kidney and its excretion into the urine. Another possibility is increased production of UII by the kidneys with diabetic nephropathy, possibly by renal tubular cells.

The kidney is one of the most important organs for the degradation of peptide hormones. Urinary IR-UII levels were similar to plasma IR-UII levels, and IR-UII clearance was much lower than creatinine clearance both in control and Type 2 diabetic patients. These findings suggest that a large part of IR-UII filtered in glomerulus from plasma is metabolized in the renal tubules and only a very small part of IR-UII is excreted in the urine. On the other hand, renal tubular cells may synthesize and secrete UII into the urine. UII and UT receptor mRNAs are expressed in the kidney [7,20]. Immunocytochemistry showed that UII was localized in the renal tubular cells [13]. Urinary excretion of UII, therefore, may not be an indicator representing the UII production in the body, but rather reflect the renal production and secretion of UII, which could be stimulated by renal damage, such as diabetic nephropathy.

Reverse phase HPLC of normal plasma showed three immunoreactive peaks, suggesting that the IR-UII in the plasma consisted of at least three molecular forms. This result is in contrast with that of Ng et al. [8], who reported a single immunoreactive peak in plasma extracts by size-exclusion chromatography. Reverse phase HPLC analysis of normal urine showed multiple immunoreactive peaks. Three of the immunoreactive peaks in the urine extract were co-eluted with those shown in the plasma extract. This finding is different from that of Matsushita et al. [7], who showed a single major immunoreactive peak in the urine extract. The discrepancy in these results may be due to the difference of chromatographic methods or assay methods used. Immunoreactive peaks eluting in the positions other than authentic UII may represent the UII precursor or UII precursor fragments. We could not deny the possibility, however, that these immunoreactive materials were generated during the extraction procedure.

It also remains to be answered why plasma IR-UII levels were elevated in Type 2 diabetic patients without renal dysfunction. Plasma IR-UII concentrations and urinary excretion of IR-UII showed no significant relations to fasting blood sugar levels or hemoglobin A1c, suggesting that high levels of blood sugar are unlikely to affect plasma IR-UII concentrations or urinary excretion of IR-UII in Type 2 diabetic patients. The recent intriguing report by Wenyi et al. showed that certain polymorphism in the UII gene was associated with Type 2 diabetes mellitus [24]. UII may therefore be related not only to endothelial cell damage in diabetes but

also to glucose metabolism, for example, in liver and skeletal muscles. Actually UII mRNA is expressed in the liver together with UT receptor mRNA [21].

The present study has shown that Type 2 diabetes mellitus itself is a factor to elevate plasma IR-UII levels, and accompanying renal failure is another independent factor for the increased plasma IR-UII levels in Type 2 diabetic patients. Furthermore, urinary excretion of IR-UII may not be an indicator representing the UII production in the body, but rather reflect the renal production and secretion of UII, which may be enhanced in advanced diabetic nephropathy.

Acknowledgments

We are grateful to Ms. Kikuchi for her secretarial and technical assistance. This study has been supported partly by Grant-in-aid for Scientific Research (C) (No. 13671094) (to KT) and (B) (No. 14370217) (to YI) from the Ministry of Education, Science, Sports and Culture of Japan.

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VI. ウロテンシン

基礎研究の進展

ウロテンシン測定法

Measurement of urotensin

戸恒和人[1]　高橋和広[2]　村上　治[3]

Key words：ウロテンシン，ラジオイムノアッセイ，腎不全，糖尿病，心不全

はじめに

ウロテンシン(urotensin)は，大部分の読者にとってなじみの薄い物質であろうと思われるが，実は古くて新しいペプチドホルモンである．30年前，硬骨魚の脊髄尾部にある神経内分泌器官urophysisから分離同定された[1]．当初からウロテンシンIとウロテンシンIIの存在が知られており，ウロテンシンIはcorticotropin releasing factor(CRF)様の作用をもつペプチドとされてきた．1995年Vaughanら[2]によりヒトを含む哺乳類でのウロテンシンIホモローグとしてウロコルチン(urocortin：UCN)が同定された．ウロコルチンは血管拡張作用および心臓保護作用などを有し，ストレスを下げる物質と理解されている．ウロコルチンは更にウロコルチンIIおよびウロコルチンIIIが同定されている．

一方，ウロテンシンIIは永らく塩類の排泄調節に関係する魚のホルモンとして理解され，臨床医学の世界では注目されていなかったが，1999年にAmesら[3]がreverse pharmacologyの手法を用いて，オルファンレセプターであるGPR14のリガンドがウロテンシンIIであることを発見し，同時にウロテンシンIIがラット胸部大動脈に対してエンドセリンよりも強力な血管収縮作用を示すこと，またヒトの動脈硬化病変部に発現を認めたことを報告し，注目されることとなった．更にウロテンシンIIには細胞増殖を促すgrowth factorとしての作用があること，心臓に対してinotropic作用があることなどが報告され，ウロテンシンIIはエンドセリンやアンジオテンシンIIなどと同じく，心血管・腎臓の病態と障害の進展に深くかかわっているのではないかと考えられるようになった．

著者らはラジオイムノアッセイ(RIA)法によるウロコルチンIIIおよびヒトウロテンシンIIの測定法を開発し，ヒト血液中および組織中にウロコルチンIII様免疫活性(UCNIII-LI)およびウロテンシンII様免疫活性(UII-LI)が存在するか否かを検討した．その結果，ヒト血液中にUCNIII-LIが存在することを見いだした[4]．更に，ウロテンシンIIはヒト血液中に循環ホルモンとして存在すること，腎不全(**図1**)[5]，糖尿病[6]などの病態で血中濃度が上昇することを見いだし，報告してきた．現在では，重症な心疾患[7-10]や門脈圧亢進を伴う肝硬変患者[11]などでも血中濃度が上昇することが報告されている．また，ウロテンシンIIがヒトの尿中に排出されていることが報告されている[12]．中枢および末梢組織での検討では，ウロテンシンIIのmRNA

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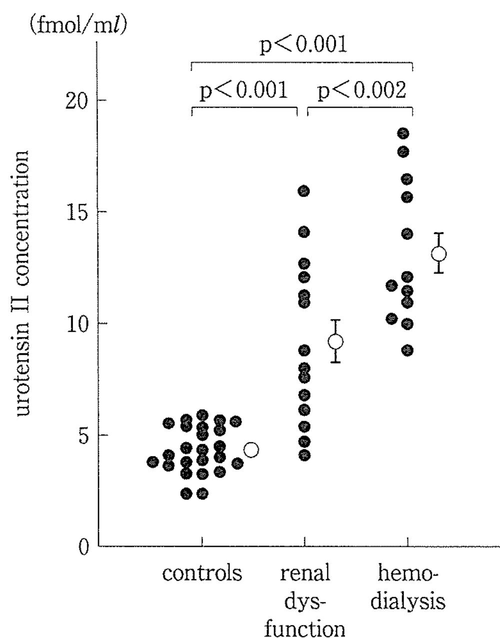

図1 血中ウロテンシンII濃度(文献[5]より引用改変)
controls: 健常者, renal dysfunction: 非透析慢性腎不全患者, hemodialysis: 透析患者.

の発現がほとんどすべての組織,臓器にみられる[5]のに対し,蛋白レベルでの発現は免疫染色法による検討で,脳,心臓[13],腎臓[12],腎腫瘍組織で発現が認められるも,RIA法を用いた検討では,各組織でのウロテンシンIIの濃度は非常に低く[14],SW-13副腎皮質癌細胞の培養液中からUII-LIが検出されているのみである[15].

本稿では,ウロテンシンIIの測定法を主体に概説する.

1. ウロテンシンIIの測定法

ウロテンシンIIの測定にはRIA法とELISA法の2種類での測定報告がみられる.

a. RIA法

RIA法は各研究者が独自に開発したものが主に使われているが,キットとしても市販されている.本稿では,著者らが使用している一般に第二抗体法と呼ばれる方法について述べる.

1) サンプルの採取

血液サンプルは静脈血を採血し,EDTA・アプロチニン加チューブに移し4℃にて冷却遠心し,血漿を分離して凍結保存する.組織は摘出後すぐに抽出するか,ドライアイスまたは液体窒素で凍結し,−80℃にて保存する.

2) 抽　出

血漿,尿および培養液は夾雑物の除去と濃縮を目的として,セパックC18カートリッジ(Sep-Pak C18 cartridge, Waters社)を用いて抽出する.

組織は1M酢酸中で100℃煮沸後,ポリトロンまたはガラス・テフロン管を用いてホモジネートし,メタノールを加えて超遠心する.上清を分離し空気流下に乾固する.BSA加酢酸液で再溶解し,セパックC18カートリッジを用いて抽出する.

セパックC18カートリッジは前処置としてメタノール,アセトニトリルおよび4%酢酸各10 mlで洗浄する.サンプル3 mlに対し4%酢酸6 mlを加えて酸性化しカートリッジにロードする.4%酢酸10 mlで洗浄後2 mlの溶出液(アセトニトリル:水:トリフルオロ酢酸=60:40:0.1)で溶出し,乾固する.500 μlのアッセイバッファーで再溶解しRIAに供する.ここで用いた溶液の詳細は文献[5,6]を参照されたい.

尿,組織抽出液および細胞培養液なども同様に抽出する.本方法での回収率は血液サンプルで95%以上である.

3) RIA

著者らが用いているRIAの手順を概説する.アッセイバッファーで再溶解したサンプル200 μlに対し抗ウロテンシンIIウサギ抗体(ペプチド研究所,ロットNo.992-500601)の希釈溶液(1,500倍)100 μlを加え4℃で48時間インキュベートする.^{125}I-urotensin II(アマシャム社)溶液100 μl(3,000 cpm/100 μl)を加え,更に48時間のインキュベーションを行い,100 μlの5%抗ウサギIgGヤギ抗体と500 μlの10%ポリエチレングリコール水溶液を加える.5時間後4℃, 3,000×gで30分遠心して上清を除去し,沈殿の放射線量をガンマカウンターで測定する.

本RIA法で検出可能な最小量は2 fmol/tubeである.

b. ELISA法

Ngら[8]は,ペニンスラ社製のELISAキットを用いて心不全患者の血中ウロテンシンII濃度を

測定して報告している．著者らはELISA法での測定経験はないので文献[8]を参照されたい．

2. UII-LIの組成

RIA法であれELISA法であれ抗体を用いる方法では，本来のウロテンシンII以外にもUIIの前駆体やその分解産物など，UIIと構造上類似する物質も含めてウロテンシンIIとしてカウントしてしまう性質をもつ．したがってUII抗体を用いて測定している場合，測定している物質をウロテンシンII様免疫活性(UII-LI)と呼ぶことが多い．

UII-LIがどのような分子型から構成されているかは，Gelクロマトグラフィーまたは高速液体クロマトグラフィー(HPLC)により検討されている．Ngら[8]はヒト血液中のUII-LIをGelクロマトグラフィーで検討し，ヒトウロテンシンIIに一致する単一ピークを報告している．Takahashiら[15]は，SW-13副腎皮質癌細胞の培養液中に含まれるUII-LIをHPLC法で検討し，3つの成分からなることを見いだし，Gelクロマトグラフィーでの検討から，そのほとんどが11個のアミノ酸からなるヒトUIIよりも大分子量の物質であることを報告している．

3. ウロテンシンIIの濃度

ウロテンシンIIの臨床的意義を明確にするには，各種疾患での血液・組織中のUII濃度測定が必要となる．前述のRIA法を用いた著者らの測定では，健常成人のUII濃度は平均4.4 fmol/*ml*であり，Richardsら[7]の報告では1.9 fmol/*ml*，Ngら[8]の報告では6.6 fmol/*ml*，Dschietzigら[9]の報告では60-80 fmol/*ml*と測定者により差がみられる．Hellerら[11]は2.6 pmol/*ml*と報告しているが，他の報告者に比して1,000倍もの高値であり，測定法または抽出法などに問題があるものと思われる．

著者ら[5]は非透析慢性腎不全患者の血中ウロテンシンII濃度が2倍，透析患者で3倍に上昇していることを報告した(**図1**)．透析の前後で濃度は変化せず，心房性ナトリウム利尿ペプチド(ANP)とは異なり，血中ウロテンシンII濃度は短期での体液量の変化は反映しないと考えられた．腎不全のない糖尿病患者でも血中ウロテンシンII濃度の上昇が認められ[6]，ウロテンシンIIの血中濃度の上昇は腎臓からの排泄の低下によるのではなく，これらの病態において産生が増加していることが示唆される．心不全患者でも2-3倍に上昇すると報告されている[7,8]が，上昇しないとの報告もみられる[9]．心不全患者の血中ウロテンシンII濃度は重症度に相関するとの報告がある一方で，重症度と関係しないとの報告もあり[8,9]，明確な答えは将来の詳細な研究を待たねばならない．

また，Hellerら[11]は肝硬変患者で約3倍の濃度上昇を報告している．Matsushitaら[12]は高血圧患者でウロテンシンIIの尿中排泄が増加していることを報告している．また，Thompsonら[16]は脊髄液中のウロテンシンII濃度は血中濃度に比して約15%低いことを報告している．

ヒト血中に存在するウロテンシンIIの産生部位は，エンドセリンと同様に血管内皮細胞が考えられるが，いまだ確定していない．ヒトの組織内ウロテンシンII濃度は，脳皮質，心臓，腎臓で検出限界以下であり，一部の副腎腫瘍組織でウロテンシンIIが検出されているのみである．

4. ウロコルチンの測定

血液および組織中のウロコルチンの測定は2つのグループから報告されている．Watanabeら[17]はRIA法にて血液中ウロコルチン濃度を測定し，健常成人男性で平均3.6 fmol/*ml*，女性で2.7 fmol/*ml*，妊娠女性で2.7-3.7 fmol/*ml*であったと報告している．また，Ngら[18]は化学発光を用いたELISA法にて心不全患者の血中濃度を検討し，健常者17.3 fmol/*ml*に対し，心不全患者は43.6 fmol/*ml*と有意な高値を示したことを報告している．各方法の詳細については文献[17,18]を参照されたい．

おわりに

ウロテンシンIIは多彩な生理作用を有するペプチドホルモンであり，これまで述べてきたように，心不全，腎不全，動脈硬化，糖尿病やあ

る種の腫瘍の病態に関与している可能性が示唆される．ウロテンシンIIの血中濃度の上昇がこれらの病態の原因であるのか結果であるのか，今後更なる検討が必要である．ウロテンシンIIの病態生理学的役割をより詳細に解明するには，多くの症例で血中濃度を測定し，結果を集積していく必要がある．しかしながら，血液中に含まれるウロテンシンIIの濃度は非常に低く，現在の測定法では感度の面から，なお大量のサンプル容量を必要としており，負荷テスト時などでの頻回の採血，測定などには困難が多い．少量のサンプルで測定が可能な，より簡便で高感度な測定法の開発が待たれる．今回は簡単に触れただけであるが，ウロコルチンについても全く同じ状況にあり，これからの研究課題といえよう．

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J Hum Genet (2004) 49:115–122
DOI 10.1007/s10038-003-0118-z

ORIGINAL ARTICLE

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Linkage and association of childhood asthma with the chromosome 12 genes

Received: 1 September 2003 / Accepted: 19 November 2003 / Published online: 7 February 2004
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Abstract Several studies have shown linkage of chromosome region 12q13–24 to bronchial asthma and related phenotypes in ethnically diverse populations. In the Japanese population, a genome-wide study failed to show strong evidence of linkage of this region. Chromosome 12 genes that showed association with the disease in at least one report include: the signal transducer and activator of transcription 6 gene (*STAT6*), the nitrogen oxide synthetase 1 gene (*NOS1*), the interferon γ gene (*IFNG*), and the activation-induced cytidine deaminase gene (*AICDA*). To evaluate the linkage between chromosome 12 and childhood asthma in the Japanese population, we performed sib-pair linkage analysis on childhood asthma families using 18 microsatellite markers on chromosome 12. To investigate association between chromosome 12 candidate genes and asthma, distributions of alleles and genotypes of repeat polymorphisms of *STAT6*, *NOS1*, and *IFNG* were compared between controls and patients. Single nucleotide polymorphism of *AICDA* was also investigated. Chromosome region 12q24.23–q24.33 showed suggestive linkage to asthma. The *NOS1* intron 2 GT repeat and *STAT6* exon 1 GT repeat were associated with asthma. Neither the *IFNG* intron 1 CA repeat nor 465C/T of *AICDA* showed any association with asthma. Our results suggest that *NOS1* and *STAT6* are asthma-susceptibility genes and that chromosome region 12q24.23–q24.33 contains other susceptibility gene(s).

Keywords Childhood asthma · Linkage · Association · Polymorphism · NOS1 · STAT6 · IFNG · AICDA

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Introduction

Bronchial asthma is an inflammatory disease of the airways characterized by airway obstruction and increased airway responsiveness. Asthma is an etiologically complex disease and develops by the interaction of multiple genes and environmental factors. Genome-wide

linkage studies have identified a number of autosomal regions providing evidence of linkage to asthma, atopy, eosinophilia, and/or other associated phenotypes (CSGA 1997; Daniels et al. 1996; Dizier et al. 2000; Haagerup et al. 2002; Hakonarson et al. 2002; Laitinen et al. 2001; Ober et al. 2000; Wjst et al. 1999; Xu et al. 2001a; Xu et al. 2000; Xu et al. 2001b; Yokouchi et al. 2000). Some of these studies (CSGA 1997; Dizier et al. 2000; Haagerup et al. 2002; Wjst et al. 1999; Xu et al. 2001a; Xu et al. 2000; Yokouchi et al. 2000) and those focused on a single chromosome (Barnes et al. 1999; Barnes et al. 1996; Kruglyak et al. 1996; Malerba et al. 2000; Nickel et al. 1997; Wilkinson et al. 1998; Raby et al. 2003) suggested linkage of chromosome 12q regions to asthma or related phenotypes in diverse populations.

In a genome-wide linkage analysis of mite-sensitive Japanese childhood asthma, the 110–145 cM region from the pter (the telomere of the short arm) showed maximum logarithm of odds score (MLS) more than 1.0 with the highest MLS of 1.92 at 111.9–125.3 cM (Yokouchi et al. 2000). The highest MLS did not reach the value of "significant" (MLS = 3.6) or "suggestive" (MLS = 2.2) linkage to the disease (Lander and Kruglyak 1995). The region in which MLS exceeded 1.0 was roughly overlapped by those of studies on Afro-Caribbean, French, and British populations (Barnes et al. 1996; Dizier et al. 2000; Wilkinson et al. 1998). To establish the linkage between asthma and chromosome 12 region, evidence of the suggestive linkage must be replicated using a different set of samples from the same population (Lander and Kruglyak 1995).

Candidate genes of chromosome 12q15–q24 include the signal transducer and activator of transcription 6 gene (*STAT6*), interferon-γ (*IFNG*), stem cell factor (*SFC*), leukotriene A4 hydrolase (*LTA4H*), insulin-like growth factor (*IGF1*), β-subunit of nuclear factor-Y (*NFYB*), B-cell translocation gene 1 (*BTG1*), and nitrogen oxide synthetase 1 (*NOS1*) (Barnes et al. 1996; Dizier et al. 2000; Wjst et al. 1999). Of these, *STAT6*, *NOS1*, and *IFNG* were investigated with case-control studies and showed positive association with asthma in at least one study. Gao et al. (2000b) demonstrated the association of the single nucleotide polymorphism (SNP) 2964G/A of *STAT6* with adult asthma in Japanese populations. However, this association was not replicated in later studies on German/Swedish (Duetsch et al. 2002) or Japanese populations (Tamura et al. 2001). Instead, a GT repeat polymorphism in exon 1 was associated with eosinophil count in the German/Swedish study and with allergic diseases in the Japanese study. A dinucleotide repeat marker in *NOS1* was also reported to be associated with the disease in the British population (Gao et al. 2000a). An association between a SNP in *NOS1* and eosinophil count was also shown in German/Swedish patients (Immervoll et al. 2001). Hyden et al. (1997) reported that no polymorphism in the *IFNG* was associated with atopic asthma, whereas an association between the GT repeat in intron 1 of *IFNG* and childhood asthma was suggested in the Japanese population (Nakao et al. 2001). Heinzmann et al. (2000a) screened polymorphisms in *SCF*, *STAT6*, *TR2* (thyroid receptor 2), and *LTA4H* and found two polymorphisms in *SCF* and one in *TR2* in the German population. They found no evidence of linkage or association of these genes with atopy.

All of the above-mentioned studies were based on case-control design. On the other hand, using the transmission disequilibrium test (TDT), Noguchi et al. (2001) reported that the activation-induced cytidine deaminase gene (*AICDA*) was associated with childhood asthma in the Japanese population. The *AICDA* gene is located in the short arm of chromosome 12, where linkage has never been suggested. They selected this gene as a candidate gene for asthma because deficiency of *AICDA* resulted in low IgE production, thereby the variations of the gene might be responsive to atopy. More recently, Isidoro-García et al. (2003) reported a case-control study on the same SNP of *AICDA* gene in the Spanish population. They failed to show the association of this SNP with the disease.

Among candidate genes of chromosome region 12q15–q24, *STAT6*, *IFNG*, and *NOS1* have been suggested to be associated with asthma in at least one study. Although the locus was not a suspected linkage to asthma, the *AICDA* gene showed a positive result in one study. As often seen in genetic analyses of a complex disease (Ioannidis et al. 2001), inconsistencies were noticed between the studies of chromosome 12 candidate genes for asthma. Studying other sets of samples in the same population is necessary to conclude whether a particular gene is truly associated with this complex disease.

In the present study, we investigated linkage of markers on chromosome 12 to childhood asthma in the Japanese population. We also investigated association of four candidate genes, *AIDCA*, *STAT6*, *NOS1*, and *IFNG*, with Japanese childhood asthma.

Materials and methods

Families and individuals

For linkage analysis, 18 families with affected sib pairs and one family with an affected sib trio were recruited. For the association study, 184 controls and 115 patients were genotyped. One hundred control subjects were selected in the Osaka area, Japan, as previously described (Heinzmann et al. 2000b; Mao et al. 1996), and 84 controls were selected from adult staff and student volunteers from Tohoku University School of Medicine in Sendai, Japan. Individuals with a history of treatment for asthma or eczema were excluded from controls. Forty-two patients were diagnosed at hospitals in the Sendai area, which included patients from 19 families for linkage analysis. For an association study, one patient per family was selected. Other patients were recruited as described (Heinzmann et al. 2000b; Mao et al. 1996). None of the samples were previously analyzed for chromosome 12 linkage markers, *NOS1*, *IFNG*, *STAT6*, or *AICDA*. Diagnosis of asthma of probands was made by pediatricians specializing in allergic diseases.

The criteria of asthma were two or more episodes of wheezing and shortness of breath and reversibility of the wheezing and dyspnea, either spontaneously or by bronchodilator treatment. The definition of "childhood" asthma was asthma with onset before age 15 years. Diagnosis of other family members was based on the modified ATS-DLD questionnaire (Ferris 1978). Total serum IgE was regarded as high when the level was 250 IU/ml or higher. The specific IgE against house dust mite [*Dermatophagoides pteronyssinus* (Dp)] was judged positive when the RAST score against Dp was 2 (0.70 U_A/ml) or higher. "Atopy" was defined as either having high total IgE and/or positive Dp-specific IgE. Eighteen families had an affected sibpair and one family had an affected sib trio. All patients with childhood asthma were atopic. All affected sibs and their parents were genotyped.

This study was approved by the ethics committee of Tohoku University School of Medicine.

Genotyping

DNA was extracted from peripheral blood leukocytes using the Genomic DNA purification kit (Promega, Madison, WI, USA). Chromosome 12 microsatellite markers of the Human GenePairs Primers version 9 (Invitrogen, Carlsland, CA, USA) were used for the linkage analysis. The 18 markers genotyped in this study are shown in Table 1. Information on marker order and position was obtained from LDB2000: Sequence-based Integrated Maps of the Human Genome (http://cedar.genetics.soton.ac.uk/public_html/LDB2000.html) (Wilkinson et al. 1998). The location of *AICDA* was not cited in this database and was estimated using the NCBI Human Map Viewer.

In chromosome 12 microsatellite marker analyses, PCR mixtures contained 10 mM Tris/HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 μM each of deoxynucleotide triphosphate (dNTPs), 0.25 U of rTaq DNA polymerase (TAKARA, Tokyo, Japan), 5 μM of each primer, and 10 ng of template DNA in a total volume of 10 μl. The cycle conditions were 94°C for 3 min, followed by 30 cycles of 94°C for 45 s, 57°C for 45 s, and 72°C for 1 min, with a final extension of 72°C for 10 min. The size of the PCR products was estimated using a 373XL DNA sequencer (Applied Biosystems, Foster City, CA, USA). GeneScan 500XL TAMRA labeled standard (Applied Biosystems) was used for estimation of fragment lengths.

Primers for the *NOS1* intron 2 GT repeat were as described previously (Gao et al. 2000a). One of the primers, 5'-ATAGAGCCTGTGCTGAGCCTTC, was 6-FAM labeled. The PCR mixture contained 10 mM Tris/HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 250 μM each of dNTPs, 0.5 U of rTaq DNA polymerase, 200 μM of each primer, and 10 ng of template DNA in a total volume of 15 μl. The cycle conditions were 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 40 s, with a final extension of 72°C for 10 min.

The primers for the *STAT6* exon 1 GT repeat were 5'-GGAGAAGCCGGAAACAGCGG and 5'-GTTCAAGGCTGGCCCTGCTAGC (6-FAM labeled). The PCR mixture was the same as for of *NOS1*. The cycle conditions were 94°C for 3 min, followed by 30 cycles of 94°C for 45 s, 57°C for 45 s, and 72°C for 1 min, with a final extension of 72°C for 10 min.

Primers for the *IFNG* intron 2 CA repeat were as previously described (Nakao et al. 2001). The PCR mixture was the same as for *NOS1*, except that 0.25 U of rTaq and 600 μM of each primer were used. The cycle conditions were 95°C for 5 min, followed by 25–34 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 1 min, with a final extension of 72°C for 5 min.

The 467C/T (His155His) polymorphism of *AICDA* (GeneBank AB040430) was the same polymorphism reported by Noguchi et al. (2001), where they designated this polymorphism as 7888C/T. This was genotyped using a modified TaqMan PCR method employing allele-specific amplification (Fujii et al. 2000). The common forward primer was 5'-GGCCCCGAGGAAATGAGAAAAT. The reverse primers were 5'-TCCCAGGCTTTGAAAGTTCTTTAG for the C allele and 5'-TCCCAGGCTTTGAAAGTTCTTTGA for the T allele. The TaqMan probe was 5'-FAM-AGAAGACAGTTCAGGTTCCAAATCGAGG- TAMRA -3'. The PCR mixture contained 7.5 μl of 2X TaqMan Universal PCR Master Mix (Applied Biosystems), 400 μM of each PCR primer, 0.12 μM of TaqMan probe, and 5 ng of template DNA in a final volume of 15 μl. The cycle conditions were 50°C for 2 min, 95°C for 3 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min.

Table 1 Map locations for chromosome 12 markers and genes

Locus	Kb from pter	Band	Male cM	Female cM	Averaged cM
ptr	0	p13.33	0.0	0.0	0.0
D12S372	3761	p13.33	7.5	2.5	5.0
AICDA	8468	p13.31	20.0	9.3	14.7
GATA49D12	8513	p13.31	20.1	9.4	14.7
D12S391	13246	p13.2	26.1	21.4	23.8
D12S373	18347	p12.3	32.8	35.5	34.1
D12S1042	28440	p11.23	36.9	56.2	46.5
cen	39000	q11	38.7	65.0	51.9
D12S1301	46378	q12	39.7	71.3	55.5
D12S398	56808	q13.13	44.4	87.0	65.7
STAT6	61349	q13.13	45.9	92.5	69.2
D12S1294	73218	q14.2	50.4	97.7	74.0
IFNG	73860	q14.2	51.7	100.3	76.0
D12S375	74485	q14.3	52.7	103.0	77.8
D12S1052	80438	q15	57.1	106.9	82.0
D12S1064	96885	q21.33	67.2	120.5	93.8
D12S1300	105918	q23.1	69.8	133.1	101.4
PAH	111421	q23.3	73.5	143.5	108.5
D12S2070	125845	q24.22	81.8	167.6	124.7
NOS1	127541	q24.22	82.8	174.6	128.7
D12S395	130349	q24.23	84.1	184.3	134.2
D12S392	138666	q24.32	91.9	200.3	146.1
D12S2078	140437	q24.33	98.1	201.8	149.9
D12S1045	143552	q24.33	110.7	212.9	161.8
qtr	146025	qtr	119.2	218.4	168.8

Statistical analysis

Allele frequencies of microsatellite markers were estimated from the parental chromosomes. All chromosome 12 markers were assessed by PEDCHECK (version 1.0) (O'Connell et al. 1998) for pedigree inconsistencies. There was no genotype inconsistency at any loci in all the families. Multipoint linkage analysis was conducted using GENEHUNTER 2 (Kruglyak et al. 1996). For score calculations of the sib trio, we used "all independent pairs of affected/phenotyped sibs" option of the GENEHUNTER 2 program where one sib trio yielded two pairs. In all allelic and genotypic distribution analyses, Fisher's exact P values were calculated using SPSS for Windows version 11.0 J (SPSS Japan, Japan). For multiallelic markers, the P value of association with the disease of each allele or genotype was multiplied by the number of the alleles or genotypes to compensate for multiple testing and expressed as Pc. The level of significance for the association studies was set at $P(Pc) = 0.05$.

Results

Linkage analysis of chromosome 12

Thirty-nine sibs with childhood asthma used for the linkage analysis consisted of 20 males and 19 females. Ages of the patients ranged from 1 to 14 years with the average age of 7.3 years. All affected sibs were positive

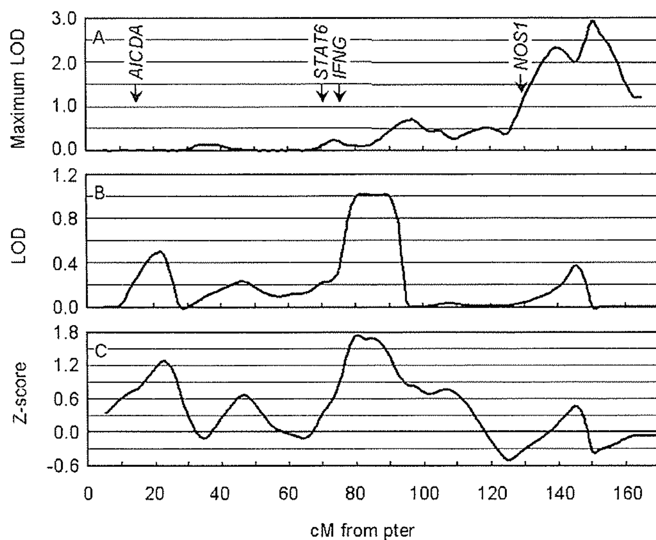

Fig. 1A–C Linkage results for asthma and total IgE level. All calculations were performed by GENEHUNTER 2. **A** Multipoint maximum likelihood (ML) estimate of identity by decent sharing for asthma affection status. **B** ML quantitative locus (QTL) variance estimation for $\log_{10}$ (total IgE level). ML QTL variance estimation (Kruglyak and Lander 1995) is performed instead of traditional Haseman-Elston (HE) QTL analysis because the values calculated with HE QTL analysis were affected by selection of sib pairs from the sib trio. **C** Nonparametric QTL analysis (Kruglyak and Lander 1995) for $\log_{10}$ (total IgE level)

for Dp-specific IgE. Thirty also showed atopic dermatitis. Results of the linkage analysis of the 18 microsatellite markers of chromosome 12 are shown in Fig. 1. A broad region from 135 cM to 160 cM exceeded 1.5 in MLS (Fig. 1A). Two peaks at 140 and 150 cM were observed, and the latter peak showed a MLS of 2.92, greater than the suggestive linkage level (Lander and Kruglyak 1995). Serum total IgE levels were $\log_{10}$ transformed and subjected to sib-pair quantitative locus (QTL) analysis. The result of maximum likelihood (ML) QTL variance estimation is shown in Fig. 1B. ML QTL variance estimation (Kruglyak and Lander 1995) was performed instead of the traditional Haseman-Elston (HE) QTL analysis, because the values with HE QTL analysis were affected greatly by the selection method of pairs from the sib trio. The highest peak of logarithm of odds (LOD) score (max = 1.02) was observed at 83 cM. No significant peaks were observed where linkage to asthma was observed. Nonparametric QTL analysis (Kruglyak and Lander 1995) showed the highest peak to be at 80 cM with a Z-score of 1.74 (P = 0.082) (Fig. 1C). The results of QTL analyses did not suggest evidence of QTL for $\log_{10}$ (total IgE level) on chromosome 12.

Association studies of genes on chromosome 12

Results of the case-control studies of chromosome 12 candidate genes are shown in Table 2. There were four types of alleles for the GT repeat of exon 1 of *STAT6* with the repeat number varying from 13 to 16 in our entire series. Comparison of allele frequencies between patients and controls showed a significant difference in the whole-allele distribution (P = 0.0054). This difference was primarily derived from the difference in the frequency of allele 3 (15 repeats). The frequency of this allele was significantly lower in the patients (Pc = 0.0044, odds ratio (OR) = 0.71, 95% confidence interval (95%CI) = 0.58–0.87). Other alleles did not show difference in frequency between controls and patients. Seven genotypes of this GT repeat were observed. The genotypic distribution differed between patients and controls (P = 0.0054). Homozygotes for allele 3 had a lower risk of asthma (Pc = 0.0035, OR = 0.718, 95%CI = 0.60–0.86).

We identified six alleles in the GT repeat polymorphism of intron 2 of *NOS1* (Table 2). The whole-allele distribution of this polymorphism of patients was significantly different from that of controls (P = 0.0082). This difference was primarily due to the difference in frequency of allele 3 (16 repeats) (Pc = 0.049, OR = 0.75, 95%CI = 0.60–0.93). Eleven genotypes of this GT repeat were observed. The whole genotypic distribution differed between patients and controls (P = 0.0019). Homozygotes for allele 3 had a lower risk for asthma than the other genotypes (Pc = 0.030, OR = 0.53, 95%CI = 0.33–0.84). There was a significant difference in the frequency of heterozygote for allele 4/5 between controls and patients (Pc = 0.028). The numbers of this genotype is small (zero in controls and six in patients), and its biological meaning is difficult to interpret.

Association of asthma was also tested for the CA repeat of the interferon γ gene (*IFNG*). We identified seven allele types and 16 genotypes of this polymorphism in the Japanese population (Table 2). There was no evidence of association between this polymorphism and asthma in our series.

We also investigated the SNP 465C/T of *AICDA*. Frequencies of the major allele were almost the same between controls and patients. Genotype distribution of controls was in Hardy-Weinberg equilibrium, whereas that of patients was not (χ^2 = 7.17, P = 0.027). We regenotyped the patients and found no evidence of typing errors. Patients showed lower CT and higher TT genotype frequencies when compared to those of controls. However, these changes did not reach statistical significance in the association study (Table 2).

Discussion

We have found evidence of a suggestive linkage between 12q24.32–33 markers (144–155 cM) and childhood asthma in the Japanese population. The region identified was very close to the region reported by Wilkinson et al. (1998), who investigated linkage to their unique asthma score in the English population. In the genome-wide analysis of Japanese mite-sensitive childhood asthma, the highest MLS (1.92) was observed between D12S78 and D12S86 (110–132 cM) (Yokouchi et al. 2000). This

Table 2 Association study of the genes on chromosome 12

	Controls (frequency %)	Asthma (frequency %)	Significance level*
STAT6 exon 1 GT repeat			
Allelic distribution			
Allele 1 (13 repeat)	77 (22.4)	72 (31.6)	*Pc* = 0.061
Allele 2 (14 repeat)	1 (0.3)	4 (1.4)	*Pc* = 0.34
Allele 3 (15 repeat)	233 (67.7)	123 (53.8)	*Pc* = 0.0044
Allele 4 (16 repeat)	33 (9.6)	29 (12.7)	*Pc* > 1.0
Overall	344 (100.0)	228 (100.0)	*P* = 0.0032
Genotypic distribution			
Allele 1/allele 1	14 (8.1)	12 (10.5)	*Pc* > 1.0
Allele 1/allele 3	46 (26.7)	41 (36.5)	*Pc* = 0.81
Allele 1/allele 4	3 (1.7)	7 (6.1)	*Pc* = 0.67
Allele 2/allele 3	1 (0.6)	4 (3.5)	*Pc* = 0.59
Allele 3/allele 3	81 (47.1)	30 (26.3)	*Pc* = 0.0035
Allele 3/allele 4	24 (14.0)	18 (15.8)	*Pc* > 1.0
Allele 4/allele 4	3 (1.7)	2 (1.8)	*Pc* > 1.0
Overall	172 (100.0)	114 (100.0)	*P* = 0.0054
NOS1 intron 2 GT repeat			
Allelic distribution			
Allele 1 (14 repeat)	2 (0.5)	1 (0.5)	*Pc* > 1.0
Allele 2 (15 repeat)	11 (3.0)	2 (0.9)	*Pc* = 0.88
Allele 3 (16 repeat)	189 (51.4)	88 (40.0)	*Pc* = 0.049
Allele 4 (17 repeat)	3 (0.8)	7 (3.2)	*Pc* = 0.27
Allele 5 (18 repeat)	126 (34.2)	97 (44.1)	*Pc* = 0.11
Allele 6 (19 repeat)	37 (10.1)	25 (11.4)	*Pc* > 1.0
Overall	368 (100.0)	220 (100.0)	*P* = 0.0082
Genotypic distribution			
Allele 1/allele 3	2 (1.1)	1 (0.9)	*Pc* > 1.0
Allele 2/allele 3	10 (5.4)	2 (1.8)	*Pc* > 1.0
Allele 2/allele 5	1 (0.5)	0 (0.0)	*Pc* > 1.0
Allele 3/allele 3	53 (28.8)	15 (13.6)	*Pc* = 0.030
Allele 3/allele 4	3 (1.6)	1 (0.9)	*Pc* > 1.0
Allele 3/allele 5	50 (27.2)	43 (39.1)	*Pc* = 0.42
Allele 3/allele 6	18 (9.8)	11 (10.0)	*Pc* > 1.0
Allele 4/allele 5	0 (0.0)	6 (5.5)	*Pc* = 0.028
Allele 5/allele 5	28 (15.2)	18 (16.4)	*Pc* > 1.0
Allele 5/allele 6	19 (10.3)	12 (10.9)	*Pc* > 1.0
Allele 6/allele 6	0 (0.0)	1 (0.9)	*Pc* > 1.0
Overall	184 (100.0)	110 (100.0)	*P* = 0.0019
IFNG intron 1 CA repeat			
Allelic distribution			
Allele 1 (12 repeat)	39 (11.0)	24 (10.6)	*Pc* > 1.0
Allele 2 (13 repeat)	209 (58.7)	110 (48.7)	*P* = 0.12
Allele 3 (14 repeat)	6 (1.7)	7 (3.1)	*Pc* > 1.0
Allele 4 (15 repeat)	91 (25.6)	78 (34.5)	*P* = 0.17
Allele 5 (16 repeat)	7 (2.0)	3 (1.3)	*Pc* > 1.0
Allele 6 (17 repeat)	0 (0.0)	1 (0.4)	*Pc* > 1.0
Allele 7 (18 repeat)	4 (1.1)	3 (1.3)	*Pc* > 1.0
Overall	356 (100.0)	226 (100.0)	*P* = 0.12
Genotypic distribution			
Allele 1/allele 1	6 (3.4)	3 (2.7)	*Pc* > 1.0
Allele 1/allele 2	19 (10.7)	8 (7.1)	*Pc* > 1.0
Allele 1/allele 3	0 (0.0)	1 (0.9)	*Pc* > 1.0
Allele 1/allele 4	8 (4.5)	9 (8.0)	*Pc* > 1.0
Allele 2/allele 2	62 (34.8)	32 (28.3)	*Pc* > 1.0
Allele 2/allele 3	3 (1.7)	0 (0.0)	*Pc* > 1.0
Allele 2/allele 4	54 (30.3)	35 (31.0)	*Pc* > 1.0
Allele 2/allele 5	6 (3.4)	2 (1.8)	*Pc* > 1.0
Allele 2/allele 7	3 (1.7)	1 (0.9)	*Pc* > 1.0
Allele 3/allele 3	0 (0.0)	2 (1.8)	*Pc* > 1.0
Allele 3/allele 4	2 (1.1)	1 (0.9)	*Pc* > 1.0
Allele 3/allele 7	1 (0.6)	1 (0.9)	*Pc* > 1.0
Allele 4/allele 4	13 (7.3)	15 (13.3)	*Pc* > 1.0
Allele 4/allele 5	1 (0.6)	1 (0.9)	*Pc* > 1.0
Allele 4/allele 6	0 (0.0)	1 (0.9)	*Pc* > 1.0
Allele 4/allele 7	0 (0.0)	1 (0.9)	*Pc* > 1.0
Overall	178 (100.0)	113 (100.0)	*P* = 0.289

Table 2 (Continued)

	Controls (frequency %)	Asthma (frequency %)	Significance level*
AICDA 465C/T			
Allelic distribution			
C	214 (58.8)	119 (57.8)	
T	150 (41.2)	87 (42.2)	
Overall	364 (100.0)	206 (100.0)	*P* = 0.860
Genotypic distribution			
C/C	63 (34.6)	41 (39.8)	
C/T	88 (48.4)	37 (35.9)	
T/T	31 (17.0)	25 (24.3)	
Overall	182 (100.0)	103 (100.0)	*P* = 0.099

* All significance levels were calculated by Fisher's exact methods. In allele-by-allele or genotype-by-genotype comparison, the *P* value was corrected by multiplying the number of alleles or genotypes and expressed as *Pc*

region is a little different from Wilkinson's and our peak regions. However, if we compare the region with MLS exceeding 1.0, the three regions overlap each other—our data 130–160 cM, Yokouchi et al. 110–150 cM, and Wilkinson et al. 130–160 cM. In an analysis of the Danish population, linkage with an MLS of 1.58 to atopic asthma at D12S392 (146 cM) was reported (Haagerup et al. 2002). More recently, linkage to airway responsiveness was also suggested at 147 cM by the Childhood Asthma Management Program (CAMP) study (Raby et al. 2003). Thus, all these studies shared a region from 140 to 150 cM. Several studies have reported linkage between asthma and the different regions of chromosome 12. The Collaborative Study on the Genetics of Asthma reported evidence of linkage to asthma of the 12q22 region. This study analyzed Caucasians, African Americans, and Hispanics and found a linkage peak at D12S2070 (125 cM) in Hispanics (CSGA 1997; Xu et al. 2001a). When the condition of loci of chromosome 14 was considered, this linkage peak became broader and greater and the region with an LOD score greater than 1.0 extended from 120 cM to the telomere (Xu et al. 2001a). The region detected in Barbados families was located at 12q21.1 (90 cM), which is clearly different from those found in the Japanese and English (Barnes et al. 1999; Barnes et al. 1996). Linkage to asthma of this region has also been suggested in German and Swedish populations (Wjst et al. 1999). A suggestive linkage (MLS = 2.81) for asthma was reported at a region around D12S390 (65 cM) in the Italian population (Malerba et al. 2000). The region was close to the region suggested by a CAMP study (Raby et al. 2003). In a founder population, Hutterites, Ober et al. (1998) suggested linkage of D12S375 (80 cM) to asthma in an earlier study, a finding not replicated in a more recent study (Ober et al. 2000). Chromosome 12 showed no evidence of linkage to asthma in Finnish families (Laitinen et al. 2001). A genome-wide study of the French population failed to detect a linkage to asthma but detected linkage to eosinophilia around the 130 cM region (Dizier et al. 2000). There was no evidence of

linkage to asthma-related phenotypes of chromosome 12 markers in the Chinese population, which is ethnically close to the Japanese population (Xu et al. 2001b).

In combination, the results of our and previous studies suggest that several genes located in 12q24–q33 are likely to be responsible for susceptibility to asthma. Some susceptibility genes of this region may be shared by several populations, and others may not. The MLS at the position of *NOS1* (128 cM), an association with which was detected in this study, was less than 1.0. The *NOS1* locus was 20 cM apart from the peak of the MLS. Thus, the region we detected in the present study most likely contains other asthma-susceptibility gene(s). In the 12q24 region, more than a hundred genes are listed in the Human Genome Map. Narrowing down the linked region by dense mapping and an intensive survey of SNPs in this region will be required to identify new susceptibility genes.

We investigated the existence of QTL for total IgE on chromosome 12 using asthma sib pairs. No region on chromosome 12 showed significant linkage to total IgE. Although type-2 error (false negative) could not be excluded due to the relatively small sample size, we speculate that loci in 12q22–q23 are asthma-susceptibility loci rather than QTLs that affect the total IgE level in the Japanese population. Using HE sib-pair QTL approaches, Barnes et al. (1996) reported that markers from 107 to 135 cM showed evidence of linkage to log (total IgE) in Barbados and Amish families. Xu et al. (2000) suggested linkage (LOD score = 2.73) to log(total IgE) of the region from *PAH* (108 cM) and D12S2070 (125 cM) in the Danish population using variance-component linkage analysis. Analysis of total IgE as a dichotomous trait (high versus normal IgE) was also performed by Nickel et al. (1997). They performed TDT in German children and presented evidence of association between markers located from 75 to 108 cM and high total IgE. On the other hand, many studies have shown negative linkage for total IgE. These include studies of the Australian population by Daniels et al. (1996), the Germans by Heinzmann et al. (2000a), the Germans and Swedish by Wjst et al. (1999), the Hutterites by Ober et al. (2000), the Chinese by Xu et al. (2001b), the French by Dizier et al. (2000), the Finnish by Leitinen et al. (2001), the Italians by Malerba et al. (2000), and the Danish by Haagerup et al. (2002). This inconstancy suggests that the relative importance of chromosome 12 loci in controlling total IgE varies from population to population.

We detected association of *STAT6* exon 1 GT repeat with Japanese childhood asthma. Our results clearly show association of the 15-repeat allele (allele 3) and asthma by allelic and genotypic distribution analyses. In Tamura's study using fewer samples than the present study, the number of heterozygote of allele 1 and allele 3 was significantly higher in patients with "allergic diseases" and that of homozygotes of allele 3 was lower in the patients with marginal statistical significance (Tamura et al. 2001). This observation was further confirmed by our present results: a change in the frequency of allele 3 homozygotes is more essential than that in the frequency of allele 1/allele 3 heterozygotes. Allele 3 appears to be a protective allele against the development of asthma in Japanese childhood asthma.

We also investigated the 2964G/A polymorphism in the 3'-UTR that was reported to be associated with adult asthma by Gao et al. (2000b) but failed to detect association in our samples (data not shown). Similar results were reported by Deutsch et al. (2002). These observations suggest that the *STAT-6* exon 1 GT repeat, but not 2964 G/A, is an asthma-susceptibility polymorphism. Further study is necessary to establish whether the polymorphism is functionally relevant to disease development or only a marker for the true functional polymorphism(s).

Association of the *NOS1* intron 2 GT repeat with asthma was also demonstrated in this study. Another study also describing the association of this polymorphism with asthma was carried out in the British population (Gao et al. 2000a). The 16-repeat allele of *NOS1* is a protective allele against asthma development in both British and Japanese populations, suggesting that this allele represents an old protective haplotype that evoked before the division of races. It is therefore possible that this polymorphism is associated with the disease in many ethnic groups. Moreover, Grasemann et al. (2000) have shown that *NOS1* exon 29 CA repeat in the 3'-UTR was associated with asthma in the US population. This is additional evidence that *NOS1* is a susceptibility gene for asthma in diverse populations. Again, further study is required to conclude whether these known polymorphisms are functionally relevant to the disease development or only markers for the true functional polymorphism(s).

We were unable to detect any association between *IFNG* and asthma in our series. Nakao et al. (2001) reported that allele frequency distribution differed between their 218 controls and 158 patients with atopic asthma in the Japanese population. Deviation of the distribution was primarily derived from the difference in frequency of allele 5 ($P = 0.0069$, $Pc = 0.048$). Frequencies in controls and patients of this allele were 4.8% and 1.3% respectively. In our study, the corresponding frequencies were 2.0% and 1.3% respectively. If we combine Nakao's and our data, P values become 0.012, which is not statistically significant after correction for the number of alleles ($Pc = 0.083$). More subjects are required to evaluate the significance of the association. Even if the association was confirmed, allele 5 would affect less than 5% of the population, i.e., the attributable fraction of this polymorphism would be low.

Although association of *AICDA* 465C/T (His155His) and asthma was suggested by TDT (Noguchi et al. 2001), our case-control study failed to prove the association of this polymorphism with asthma in the same population. In allelic distribution, allele frequency was essentially the same between the patient and control groups. Genotype distribution of the patients was not in

Hardy-Weinberg equilibrium. This did not result in significant difference in the genotype frequency between cases and controls. The data suggest that case samples were not well representative of the homogeneous population. We genotyped another set of 94 cases with this SNP. Genotypes CC, CT, and TT were 25, 50, and 19 cases respectively. Genotype frequency of the new set of patients was in Hardy-Weinberg equilibrium and not significantly different from the control value. Although we cannot exclude *AICDA* completely as a candidate gene for asthma, the effect of the 465C/T polymorphism on susceptibility to asthma was not as strong as the repeat polymorphisms of *STAT6* or *NOS1*.

In conclusion, we demonstrated that *STAT6* and *NOS1* loci are associated with childhood asthma in the Japanese population and showed evidence of "suggestive" linkage between region 12q24.23–q24.33 and asthma. This chromosome region most likely contains as yet unidentified asthma susceptibility gene(s).

Acknowledgement We thank all members of the families and volunteers who participated in this study for their kind cooperation. We also thank Ms. Kumi Kato for excellent technical assistance. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan, and grants from the Ministry of Health, Labor, and Welfare, Japan.

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American Journal of Medical Genetics 130A:40–44 (2004)

Significant Association Between Nonsyndromic Oral Clefts and Arylhydrocarbon Receptor Nuclear Translocator (ARNT)

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The etiology of nonsyndromic oral clefts (cleft lip, cleft palate, or cleft lip and palate) is still controversial, but is considered to involve both genetic and environmental factors. One of suspected environmental factors is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) found in tobacco, herbicides, contaminated soil, and food. TCDD administered during organogenesis in mice causes a high incidence of CP in fetuses. There is ample evidence that aryl hydrocarbon receptor (AHR), AHR nuclear translocator (ARNT), and cytochrome P450 1A1 (CYP1A1) are involved in TCDD metabolism. We assessed whether there is any association in the Japanese population of nonsyndromic oral clefts with single nucleotide polymorphisms (SNPs) in the *AHR*, *ARNT*, and *CYP1A1* genes using transmission disequilibrium test (TDT) and case-control study. We identified and investigated three SNPs in ARNT; 567G/C (V189V), IVS12-19T/G, and 2117C/T (P706L). Two amino acid substitutions, R554L in AHR and I462V in CYP1A1, were also investigated. In the TDT, the C allele of ARNT 567G/C was preferentially transmitted to patients ($P = 0.033$). When a haplotype consisting of 567G/C and IVS12-19T/G in ARNT was considered, the preferential transmission of the CT (567C-IVS12-19T) haplotype was observed ($P = 0.0012$). In a case-control study, a significant association of IVS12-19T/G in ARNT was observed ($P = 0.021$). The SNPs studied in AHR and CYP1A1 were not associated with the disease. Our results suggest that ARNT is involved in the development of nonsyndromic oral clefts in the Japanese population. © 2004 Wiley-Liss, Inc.

KEY WORDS: nonsyndromic oral clefts; aryl hydrocarbon receptor (AHR); AHR nuclear translocator (ARNT); cytochrome P450 1A1 (CYP1A1); transmission disequilibrium test (TDT); 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)

INTRODUCTION

Nonsyndromic oral clefts, which include cleft lip (CL), cleft lip and palate (CLP), and cleft palate (CP), are one of the most common congenital anomalies, with an incidence of 1/700 to 1/1,000 live births. Both environmental and genetic factors are thought to be involved in their pathogenesis [Murray, 2002]. Numerous studies have shown that genetic factors appear to play a significant role in the etiology of nonsyndromic oral clefts [Wyszynski et al., 1996]. However, the genetic characteristics of nonsyndromic oral clefts are complicated by an uncertain mode of inheritance, incomplete penetrance, and heterogeneity both within and among populations [Maestri et al., 1997]. Candidate genes for nonsyndromic oral clefts have been proposed based on the allelic association studies, linkage analysis and animal studies [Schutte and Murray, 1999]. Studies with knockout mice have identified putative genes that cause the cleft phenotype in the knockout mice without other physical or developmental anomalies and are expressed at critical times in tissues relevant to lip and palate development [Schutte and Murray, 1999]. Some knockout genes, such as the homeobox homologue 1 (*MSX1*), transforming growth factor-β_3 (*TGFB3*), the β_3 subunit of the gamma-aminobutyric acid receptor (*GABRB3*) genes, not only resulted in a cleft phenotype but also exhibited a significant association with human nonsyndromic oral clefts [Maestri et al., 1997; Lidral et al., 1998; Schutte and Murray, 1999; Scapoli et al., 2002], although some subsequent studies did not support these results [Murray, 2002].

One of the suspected environmental factors for the development of nonsyndromic oral clefts, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), causes a high percentage of CP in fetuses when administered during organogenesis in mice [Courtney and Moore, 1971]. TCDD is found in tobacco smoke, contaminated foods, contaminated soil, and gas exhaust from motor vehicles [Muto and Takizawa, 1989; Gullett and Ryan, 2002; Huwe, 2002]. TCDD in tobacco smoke is suspected to be involved in the development of nonsyndromic oral clefts, and maternal smoking during pregnancy has been identified as a risk factor for nonsyndromic oral clefts [Shaw et al., 1996; Lorente et al., 2000].

Aryl hydrocarbon receptor (AHR), AHR nuclear translocator (ARNT), and cytochrome P450 1A1 (CYP1A1) are involved in TCDD metabolism. TCDD binding with the AHR results in nuclear translocation and release of heat shock protein 90, followed by dimerization with ARNT, and DNA binding/

Grant sponsor: Ministry of Education, Science, Sports, and Culture of Japan; Grant number: 13470378; Grant sponsor: Ministry of Education, Culture, Sports, Science and Technology, Japan (grants-in-aid for scientific research); Grant sponsor: Japanese Millennium project.

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Received 26 June 2003; Accepted 6 October 2003

DOI 10.1002/ajmg.a.30023

Published online 27 July 2004 in Wiley InterScience (www.interscience.wiley.com)

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transactivation. The *CYP1A1* gene is one of the targets of this complex [Sogawa and Fujii-Kuriyama, 1997]. Interestingly, the AHR, ARNT, and CYP1A1 mRNAs are expressed in developing craniofacial tissue of mice and the CYP1A1 mRNA was highly induced by TCDD exposure [Abbott et al., 1999]. When pregnant mice were administered with a dose of 40 μg TCDD/kg body weight by gavage at gestation day 12.5, almost all wild-type fetus suffered from CP, whereas none of AHR-null mutant fetuses were sensitive to TCDD [Mimura et al., 1997]. ARNT-null mice are not viable past gestation day 10.5, because most knockout embryos have retarded growth as indicated by significant decreases in crown rump and head length; abnormalities include neural tube closure defect, failure of the head folds to close completely, forebrain hypoplasia, delayed rotation of the embryos, placental hemorrhaging, and visceral arch abnormalities [Kozak et al., 1997].

These observations led us hypothesize that AHR, ARNT, and CYP1A1 may be involved in the pathogenesis of nonsyndromic oral clefts. Although there is only one association study between nonsyndromic oral clefts and polymorphisms of CYP1A1 [van Rooij et al., 2001], no association study on AHR or ARNT has been reported. In the present study, we assessed whether there is any association of nonsyndromic oral clefts and SNPs in AHR, ARNT, and CYP1A1.

MATERIALS AND METHODS

Families

A total of 148 Japanese nonsyndromic oral clefts probands and their parents were recruited for this study (36 CL cases, 8 CP cases, and 104 CLP cases). Each of ten probands had an affected parent. Each of six probands had an affected sib. One proband had both affected parent and a sib. The rest of the probands (131 cases) had no affected individual in the first-degree relatives. Written and oral informed consent was obtained from all subjects. A family history was obtained from each participant to determine the presence or absence of related individuals with nonsyndromic oral clefts and other congenital anomalies. This study was approved by the Ethics Committee of Tohoku University School of Medicine. Blood samples were collected from the probands, their parents, and 189 healthy Japanese volunteers as controls.

DNA analysis

DNA was prepared from whole blood samples using a GFX DNA purification kit (Amersham Pharmacia Biotech, Buckinghamshire, England). We amplified exons 1–22 and a 1 kb promoter region of ARNT using previously identified primers by standard PCR [Scheel et al., 2002]. The amplified products were purified (Qiagen K.K, Tokyo, Japan) and directly sequenced with ABI genetic analyzer 310 (Applied Biosystems, Foster City, CA). The sequences were analyzed by computer program ATGC (GENETYX, Tokyo, Japan).

Genotyping of SNPs in AHR, ARNT, and CYP1A1 was performed with a unique allele-specific TaqMan PCR method [Fujii et al., 2000] using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Primers used for TaqMan PCR are shown in Table I. PCR mixtures containing 7.5 μl of 2× TaqMan Universal PCR Master Mix (Applied Biosystems), 0.4 μM each of PCR primer, 0.12 μM of TaqMan probe, and 10 ng of template DNA in a final volume of 15 μl were subjected to using the following thermal cycling program: 50°C for 2 min, 95°C for 3 min, and 45 cycles of 95°C for 15 sec and 60°C for 1 min.

The standard single-allele transmission disequilibrium test [Spielman et al., 1993] and haplotype TDT of ARNT were carried out by GENEHUNTER 2 [Kruglyak et al., 1996]. Pairwise linkage disequilibrium in each SNPs was estimated as D′ [Lewontin, 1964] and r^2 [Hill and Robertson, 1968]. Allele frequencies in patients and controls were compared by contingency Chi-square test. Fisher's exact *P* values were calculated using SPSS software version 11.0J (SPSS Japan, Inc., Tokyo, Japan).

RESULTS

Sequencing of ARNT in 17 cases, that had positive family history in the first-degree relatives, identified three SNPs

TABLE I. Primer and TaqMan Probe Sequences for the TaqMan Allele-Specific Amplification

(A) Allele-specific amplification of 1661A/G in the *AHR* gene	
Forward primer for A-allele	5′-GCATTGATTTTGAAGACATCCA-3′
Forward primer for G-allele	5′-GCATTGATTTTGAAGACATCCG-3′
Reverse primer	5′-CCATACAGCTTGAGTTCAGAGC-3′
TaqMan probe[a]	5′-CGGATGAAATCCTGACGTATGTCCAAGA-3′
(B) Allele-specific amplification of 567G/C in the *ARNT* gene	
Forward primer for G-allele	5′-GACAGGCAGGGTGGTGTATGCG-3′
Forward primer for C-allele	5′-GACAGGCAGGGTGGTGTATGCC-3′
Reverse primer	5′-GCTGCCAAACCATTCAGACTG-3′
TaqMan probe[a]	5′-CTGACTCCGTGACTCCTGTTTTGAACCAG-3′
(C) Allele-specific amplification of IVS12-19T/G in the *ARNT* gene	
Forward primer	5′-AACTCAGGAGGTGGAGGT-3′
Reverse primer for T-allele	5′-GAGTTCCTAGAATACAGAAAGAAA-3′
Reverse primer for G-allele	5′-GAGTTCCTAGAATACAGAAAGATC-3′
TaqMan probe[a]	5′-CAGTGAGCCAAGATCGCGC-3′
(D) Allele-specific amplification of 2117C/T in the *ARNT* gene	
Forward primer for C-allele	5′-CTTCCTTGCTGTATTTCTAGCTAC-3′
Forward primer for T-allele	5′-CTTCCTTGCTGTATTTCTAGCTTT-3′
Reverse primer	5′-ACACCCACACCCTCTGCTGT-3′
TaqMan probe[a]	5′-AGACTGGACAGACTGCAGGACAATTCC-3′
(E) Allele-specific amplification of 1384A/G in the *CYP1A1* gene	
Forward primer for A-allele	5′-CGGAAGTGTATCGGTGAGACTA-3′
Forward primer for G-allele	5′-CGGAAGTGTATCGGTGAGACTG-3′
Reverse primer	5′-AAGCACCTAAGAGCGCAGCTG-3′
TaqMan probe[a]	5′-CGTGAAGGTGGACATGACCCCCAT-3′

[a]Each TaqMan probe was labeled with 6-carboxyfluorescein (FAM, reporter dye) at the 5′-end and 6-carboxy-tetramethyl-rhodamine (TAMRA, quenching dye) at the 3′-end.

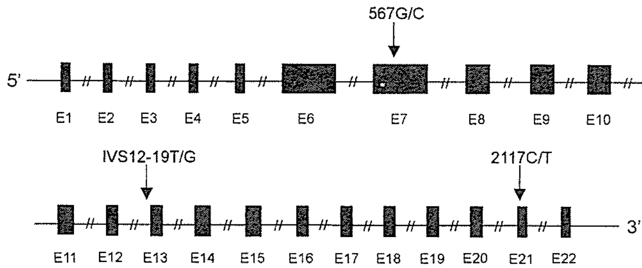

Fig. 1. The schematic structure of the human *ARNT* gene [Scheel et al., 2002]. Genetic variations are indicated by arrows. E, exon.

with the following allelic variations: 567G/C in exon 7 (GenBank accession number AJ404854), IVS12-19T/G in intron 12 (nt 16623, GeneBank accession number AJ404854), and 2117C/T in exon 21 (GeneBank accession number AJ251863) (Fig. 1). A substitution of G to C at nt 567 altered the third nucleotide of codon 189 (valine), but did not change the amino acid. A substitution of T to G at IVS12-19 did not affect consensus sequence for mRNA splicing. A substitution of C to T at nt 2117 altered the second nucleotide of codon 706 (proline), resulting in an amino acid change to leucine. All three SNPs are found in the dbSNP database (567G/C NCBI CLUSTER ID: rs no. 2228099, IVS12-19T/G rs no.3768017, and 2117C/T rs no. 2275237).

In 100 unaffected control subjects, allele frequencies of 567C, IVS12-19G, and 2117T were 0.4, 0.15, and 0.02, respectively. Two of three SNPs, 567G/C, IVS12-19T/G were subjected to TDT, whereas 2117C/T was not because of a low allelic frequency. The frequency of 2117C/T allele in 96 patients was 0.015 and not significantly different from the control value.

Pair-wise linkage disequilibrium coefficients between 567G/C and IVS12-19T/G were estimated. D′ and r^2 were 0.739 and 0.0718, respectively, both of which suggested that two SNPs were in linkage disequilibrium.

TDT was performed using 567G/C and IVS12-19T/G in ARNT, 1661A/G in AHR, and 1384A/G in CYP1A1 for 148 patient–parents trios (Table II). The 567C allele of ARNT was transmitted preferentially to children with nonsyndromic oral clefts ($P = 0.033$). When CP patients were excluded from the test, both 567C allele and IVS12-19T allele of ARNT were transmitted preferentially to the patient with the marginal significance (567C, $P = 0.054$; IVS12-19T, $P = 0.049$). Next, we tested transmission of haplotypes consisted with 567G/C and IVS12-19T/G. Among four haplotypes observed, the 567C and IVS12-19T haplotype (CT haplotype) was preferentially transmitted to the patients ($P = 0.0012$) (Table III). The 567G and IVS12-19G haplotype (GG haplotype) was transmitted to the patients less than 50% chance with the marginal significance ($P = 0.052$). Other haplotypes did not show any deviation of transmission. When CP patients were excluded, both overtransmission of CT haplotype and undertransmission of GG haplotype were significant ($P = 0.0023$ and $P = 0.023$, respectively). Other haplotypes did not show any deviation in their transmission from the parents to the patients. Association of 567G/C and IVS12-19T/G with the disease was also evaluated in the case-control samples (Table IV). None of the patients with oral clefts were homozygous for IVS12-19G, whereas 4.2% of the controls were homozygous for this allele. The exact P value for this change in total oral clefts was 0.010 and that for CL + CLP was 0.023. Decrease in frequency of homozygotes of the G allele corresponded to the undertransmission of IVS12-19G observed in the TDT (Table II). There was no difference in frequency of the heterozygote at this SNP between the cases and controls.

TABLE II. Results of TDT of Genes Involved in TCDD Metabolism

	CL + CLP + CP (148 families)					CL + CLP (140 families)			
Polymorphism	Allele	T	NT	χ^2	P	T	NT	χ^2	P
AHR (1661A/G)	A	65	59	0.29	0.59	60	57	0.08	0.78
ARNT (567G/C)	C	70	47	4.52	0.033*	64	44	3.70	0.054**
ARNT (IVS12-19T/G)	T	47	32	2.85	0.091	46	29	3.85	0.049*
CYP1A1 (1384A/G)	G	46	43	0.10	0.75	45	39	0.43	0.51

χ^2 and P values were calculated using the GENEHUNTER 2 program. CL, cleft lip; CLP, cleft lip and palate; CP, cleft palate; T, transmitted; NT, not transmitted.
*Significant.
**Marginally significant.

TABLE III. Results of ARNT Haplotype Transmission to Patients With Nonsyndromic Oral Clefts

	CL + CLP + CP (148 families)				CL + CLP (140 families)			
Haplotype	T	NT	χ^2	P	T	NT	χ^2	P
GG haplotype[a]	19	33	3.77	0.052**	17	33	5.12	0.023*
GT haplotype[b]	40	54	2.09	0.15	39	48	0.93	0.33
CG haplotype[c]	0	2	2.00	0.16	0	2	2.00	0.15
CT haplotype[d]	58	28	10.47	0.0012*	53	26	9.23	0.0023*

χ^2 and P values were calculated using the GENEHUNTER 2 program.
CL, cleft lip; CLP, cleft lip and palate; CP, cleft palate; T, transmitted; NT, not transmitted.
*Significant.
**Marginally significant.
[a]567G and IVS12-19G.
[b]567G and IVS12-19T.
[c]567C and IVS12-19G.
[d]567C and IVS12-19T.

TABLE IV. Association Between SNPs of ARNT and Patients With Nonsyndromic Oral Clefts

Name	Number	Genotype			OR (95% CI)	χ^2	P
ARNT (567G/C)		GG	GC	CC			
CL + CLP + CP	148	54	65	29	1.20 (0.69–2.09)[a]	0.401	0.821
CL + CLP	140	53	60	27	1.17 (0.67–2.07)[a]	0.332	0.853
Controls	189	72	85	32			
ARNT (IVS12-19T/G)		TT	TG	GG			
CL + CLP + CP	148	104	44	0	Could not be calculated	6.996	0.021*
CL + CLP	140	99	41	0	Could not be calculated	6.513	0.030*
Controls	189	134	47	8			

χ^2 and P values were calculated using the SPSS ver. 11.0J program.
OR, odds ratio; CI, confidence interval; CL, cleft lip; CLP, cleft lip and palate; CP, cleft palate.
[a]GG + GC versus CC.
*Significant.

The 1661A/G in AHR and 1384A/G in CYP1A1 showed no association with the disease (Table II).

DISCUSSION

Results of TDT of SNPs in the *ARNT* gene show evidence of association of this gene and nonsyndromic oral clefts in the Japanese population. The CT haplotype was transmitted to the patients more often than expected under the assumption of no association with the disease risk. This result suggests that the CT haplotype is associated with the disease-promoting nucleotide change(s). Although the significance was marginal, the GG haplotype was transmitted less often than expected. The GG haplotype may be associated with disease-protective change(s). The GT allele did not show any effect on the development of nonsyndromic oral clefts. Since the frequency of the CG haplotype was small, we could not evaluate the risk of this allele. In the case-control study, we observed significant association between nonsyndromic oral clefts and IVS12-19T/G of ARNT. None of the oral cleft patients was homozygous for IVS12-19G, whereas the frequency of the heterozygote among the patients was the same as that in the controls. This suggests that the IVS12-19G allele is a recessive protective allele against the disease. The observation is consistent with the result of the haplotype TDT that showed that the 567G and IVS12-19G haplotype was transmitted less often to the patients than 50% chance. Odds ratio and its 95% confidence interval of IVS12-19G/G could not be calculated because the number of homozygous patients was zero. To estimate these values, more patients should be analyzed.

Because neither the 567G/C nor the IVS12-19G/T haplotypes affects amino acid sequence or splicing consensus sequence, it is not likely that these SNPs are directly involved in the pathogenesis of nonsyndromic oral clefts. They are probably in linkage disequilibrium to unidentified genetic variation(s) that are responsible for the development of the disease. Alternatively, it is possible that these seemingly neutral nucleotide substitutions affect mRNA splicing and/or the gene expression as described in other genetic disorders [Wakamatsu et al., 1992; Kajihara et al., 1995]. Effects of these SNPs on splicing should be assessed to determine whether these SNPs are pathogenic of not. If these are not directly responsible for the pathogenesis of oral clefts, polymorphisms located in and near ARNT and in linkage disequilibrium to the GG haplotype or CT haplotype should be extensively examined for their effects on function and expression of ARNT.

Some of epidemiologic and embryologic studies suggest that CL with or without CP (CL + CLP) is a distinct entity from CP only [Murray, 2002]. Thus, we examined the association of SNPs in all oral clefts patients and CL + CLP patients. The results of the TDT and case-control study on ARNT were essentially the same as shown in Table II–IV. It was not possible to elucidate whether ARNT is associated with defective development of lips or palate or both.

A total of 17 patients had positive family history in the first-degree relatives. We have performed TDT analysis on the patients other than these 17 cases. The results of the TDTs were essentially the same (data not shown). There was no evidence for a different effect of ARNT between familial and sporadic cases.

Neither of two SNPs in AHR and CYP1A1 showed the association with oral clefts in this study. The observation did not preclude possible enrollment of these genes in the pathogenesis of oral clefts.

For mothers involved in agricultural activities during the month before conception and the first trimester of pregnancy, the adjusted odds ratio for carrying a fetus with malformations is 3.16 (95% confidence interval, 1.11–9.01) primarily due to an increased risk for nervous system defects, oral clefts, and multiple anomalies [Garcia et al., 1999]. Although controversial, an association between prenatal exposure to tobacco and oral clefts in humans has also been suggested in several studies [Lorente et al., 2000]. TCDD in herbicides and tobacco may increase the risk of nonsyndromic oral clefts [Wyszynski and Beaty, 1996; Garcia et al., 1999]. Interestingly, the teratogenic effect of TCDD has been reported to vary among mice of different genetic backgrounds [Courtney and Moore, 1971]. The importance of AHR–ARNT pathway in palate development has been further advocated by genetic approaches [Abbott et al., 1999].

In conclusion, our study employing TDT and case-control study on SNPs in the *ARNT* gene provides evidence, in addition to the previous AHR and ARNT knockout mice studies, for the possible involvement of the AHR–ARNT pathway in the pathogenesis of nonsyndromic oral clefts. To establish an association between the *ARNT* gene and nonsyndromic oral clefts, studies with a larger number of families from various ethnic groups may be necessary.

ACKNOWLEDGMENTS

We thank all of the members of the families and volunteers who participated in this study for their kind co-operation. We also thank Ms. Kumi Kato for excellent technical assistance.

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