Long-term Benefits of Regenerative Therapy Using FGF-2

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Abstract：Basic fibroblast growth factor (FGF-2) is one of the major candidates as a periodontal tissue regenerating agent. A series of animal studies and clinical trials have demonstrated its efficacy and safety. In the present study, we surveyed the eight-year periodontal treatment and symptom records of 79 patients who had been administered investigational drugs containing 0% (placebo; vehicle alone), 0.03%, 0.1% or 0.3% human recombinant FGF-2 (Code No. KCB-1D) in the exploratory phase II clinical trial, to evaluate the long-term benefits of regenerative therapy using FGF-2. The treatments and symptoms caused by progression of local periodontitis and those not related to periodontitis were categorized as "events" or "censored", respectively. The number of events was 14, and survival analysis (generalized Wilcoxon test) revealed that 0.3% FGF-2 significantly prolonged the time to "event" as compared with vehicle alone ($p=0.0345$). In this study, no safety problem was observed

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Key words：regenerative therapy, basic fibroblast growth factor, long-term follow up, retrospective study, survival analysis

要旨：塩基性線維芽細胞増殖因子（FGF-2：basic fibroblast growth factor）は，歯周組織再生誘導薬の有力な候補の一つと期待されており，動物実験および臨床治験によって，その有効性と安全性が明らかにされている。本研究では，KCB-1D（歯周病を対象とした遺伝子組み換えヒト型 FGF-2 の治験薬コード）を用いた探索的第 II 相臨床治験に参加した 79 名の被験者を対象として，フラップ手術時にプラセボ（0%）あるいは 0.03%，0.1%，0.3%の何れか濃度の FGF-2 を投与した被験歯の長期経過を調査した。すなわち，診療録などの診療情報から，臨床治験最終観察日から本研究調査実施日までの間（約 8 年間）に，各種濃度の FGF-2 あるいはプラセボを投与された被験歯に対して行なわれた治療や，被験歯に出現した症状の内容と年月日を調査した。そして，これらのうち，治験薬投与部位における歯周炎の悪化に起因すると判定された治療や症状をイベント，それ以外を打ち切りとして，生存時間解析を行った。その結果，発生した全イベントは 14 例で，生存時間解析の結果，0.3% FGF-2 投与群はフラップ手術を単独で施行したプラセボ群に比べてイベント発生までの期間の有意な延長が認められた（一般化 Wilcoxon 検定：$p=0.0345$）。また，本研究の観察期間を含めて FGF-2 投与の安全性に関する問題は認めなかった。

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緒　言

超高齢化社会を迎え，歯の喪失に伴う様々な QOL の低下が社会的問題となっている我国で，成人が歯を喪失する第一の原因は歯周炎である[1]。そのため，歯周炎によって失われた歯周組織を再生させることにより歯の喪失を防ぐことができれば，生涯を通じて自分の歯で咀嚼することが可能となり国民の QOL の向上，さらにはより良い全身状態の維持に寄与するものと考えられる。そこで現在，歯周炎による歯の喪失を減少させるため，重度歯周炎に対する標準的治療法となりうる歯周組織再生誘導薬の開発が強く求められている。

塩基性線維芽細胞増殖因子（FGF-2：basic fibroblast growth factor）は，歯周組織再生誘導薬の有力な候補の一つとして期待され，ビーグル犬[2,3]やカニクイザル[4]を用いた動物実験および 3，2 壁性歯槽骨欠損を有すると診断された歯周炎患者を対象とした第 II 相臨床治験[5,6]で，その歯周組織再生誘導能と安全性が明らかになってきている。これらの第 II 相臨床治験では，FGF-2 投与 36 週後の時点で，臨床的な付着を獲得しつつ，通常のフラップ手術に比べて統計学的に有意な新生歯槽骨の増加がもたらされることが証明されている。しかしながら，歯周組織再生療法の真の目的が長期的な歯の保存であることを考慮すると，歯の予後を含めた FGF-2 投与のさらに長期的な効果と安全性を検討することは大きな意義があると考えられる。そこで，本研究では，上記の FGF-2 を用いた新規歯周組織再生療法の開発に係る第 II 相臨床治験のなかでも，早期に実施され長期の術後観察が可能である探索的試験[5]に参加した被験者を対象として，各種濃度の FGF-2 あるいはプラセボを投与した歯の長期経過を調査し，歯周炎に対する FGF-2 を用いた歯周

組織再生療法の長期的な効果と安全性を検討した。

材料および方法

1. 被験者

対象は，KCB-1D（歯周病を対象とした遺伝子組み換えヒトFGF-2の治験薬コード）探索的試験[5]で試験対象の3壁もしくは2壁性の骨欠損形態を有すると診断された部位（各被験者につき1部位）に治験薬が投与された被験者79名とした。同治験は二重盲検・多施設共同・無作為化・プラセボ対照のデザインで，医薬品の臨床試験の実施に関する基準（GCP：Good Clinical Practice）遵守下で，科研製薬株式会社の依頼に基づき2001年～2004年に実施された。被験歯には，ハイドロキシプロピルセルロース（HPC）を基材としたプラセボ（0%），0.03%，0.1%，0.3%の何れかの濃度のFGF-2を含有する治験薬がフラップ手術時に単回投与され，それぞれの歯周組織再生状態が比較・検討され，0.3% FGF-2投与群で統計学的に有意な歯槽骨の増加が認められている[5]。

2. 研究組織および研究デザイン

KCB-1D探索的試験[5]を実施した13施設の研究責任者が臨床研究施設の被験者の診療録などの診療情報から，臨床治験最終観察日から本研究調査実施日までの間に被験歯にみられた歯周病の再発や予後に関わる各種事象の発生日と発生状況を追跡可能な限り調査した。その後，研究代表者（村上伸也）が各施設の研究責任者から匿名化されたデータの提供を受け，各種濃度のFGF-2あるいはプラセボが投与された被験歯における各種事象の発生頻度や程度を比較・解析した。なお，本研究に必要なKCB-1D探索的試験[5]の成績は，科研製薬株式会社から研究代表者に提供された。

3. 調査実施手順

各臨床研究施設の研究責任者あるいは主治医等の研究分担者は，所属研究施設の診療録等の診療情報より，治験薬投与36週後のKCB-1D探索的試験[5]の最終観察日から本研究調査日までの期間に被験歯（治験薬を投与した歯）に対して行った治療もしくは被験歯に生じた事象（以下，治療等）のうち(1)～(6)に該当するものとその時期（年月日）を調査した。そして，さらに，(1)～(5)については，当該治療等の発生が治験薬投与部位の歯周炎の悪化に起因するか否かを各臨床研究施設の研究責任者あるいは研究分担者が判定した。調査内容は調査票に記載され，匿名化されて研究代表者へ提出された。なお，各施設の研究責任者あるいは研究分担者の判定の妥当性に関しては別途設置した委員会で評価・確認された。

(1) 抜歯（治験薬投与部位を有する歯根の抜去を含む）
(2) 歯周組織再生療法（エナメルマトリクスタンパク：EMDを用いた歯周組織再生療法，歯周組織再生誘導法：GTR法等）
(3) 歯周組織再生療法を除く歯周外科手術
(4) 積極的な介入をした非外科的歯周治療（歯肉縁下の処置を目的としたスケーリング・ルートプレーニング，局所抗菌薬投与等）
(5) その他，歯周炎の進行が原因となって生じた事象（逆行性歯髄炎等）
(6) 異常な歯周組織の治癒（歯肉増殖等）が疑われる所見

4. 統計解析

上記の(1)～(5)の治療等うち，治験薬投与部位の歯周炎の悪化に起因する治療等であると判定されたものをイベント，それ以外を打ち切りとして，生存時間解析を行った。なお，観察期間内に同一被験歯に治療等が複数存在する場合には，最初に発生した治療等をイベントもしくは打ち切りとして扱った。また，観察期間内に治療等が認められなかった被験者のうち，現在は来院していない被験者は診療情報が最後に得られた日を打ち切り日に，そして，現在も来院している被験者は調査実施日を打ち切り日とした。

5. 倫理的対応

本研究は，「臨床研究に関する倫理指針（平成20年7月31日厚生労働省告示第415号）」を遵守して実施され，実施前に各研究施設の倫理委員会もしくはそれに相当する組織の承認を得た。本研究は被験者に対する介入を伴わない既存の診療情報のみを用いた観察研究であるため，被験者に対するインフォームド・コンセントは各研究施設が必要と判断した場合にのみ取得した。そして，インフォームド・コンセントの取得が必要ではないと判断された場合においては，本研究の概要を各臨床研究施設のホームページ等で公開した。

結　果

1. 被験者の構成

被験者構成を図1に示す。治験薬投与が行われた被験者79名の中で，25名は転院等によって調査実施日までの診療情報の一部が得らなかったため治療等が発

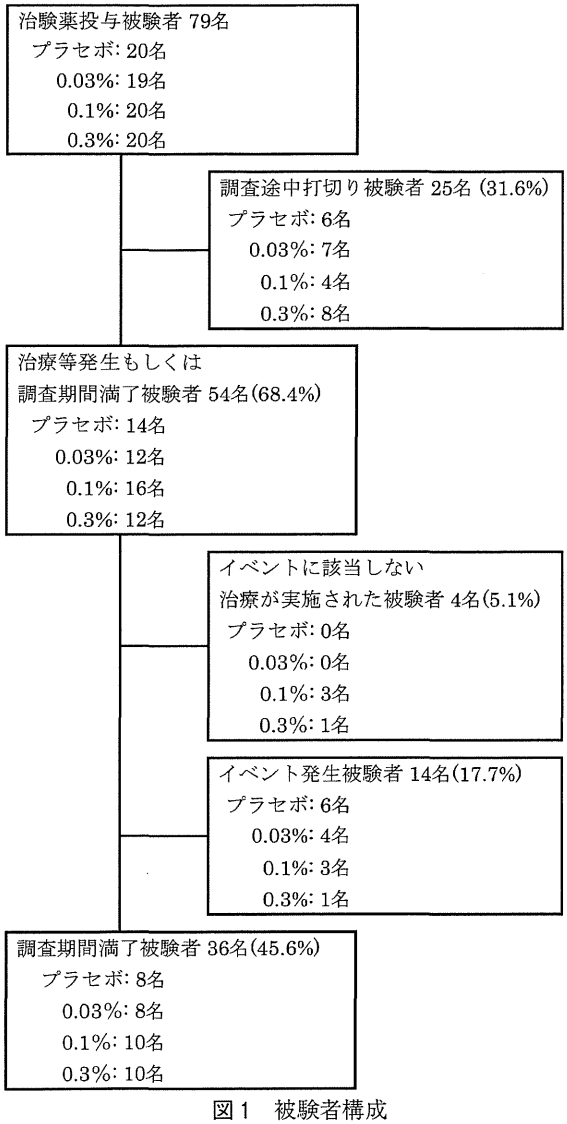

図1 被験者構成

生したか否かを十分に判断できない調査途中打ち切り被験者であった。残りの54名の中で，イベント発生被験者は14名，イベントに該当しない治療等（治療計画上の積極的な抜歯，根尖性歯周炎のための歯根端切除術等）により打ち切りとなった被験者は4名，そして，調査実施日までイベントの発生やイベントに該当しない治療の実施がなかったことが診療情報から確認できた調査期間満了被験者は36名であった（図1）。

2. 各群の被験者，被験歯および試験部位の特性と観察期間

KCB-1D探索的試験[5]参加時の被験者の性別や年齢，被験歯の歯種および試験部位の骨吸収等の臨床所見に特記すべき群間の偏りは認められていない。そして，治験薬投与日から最後に被験歯を観察した日までの期間も，全ての群で同程度であった（表1）。

表1 各群の治験薬投与日から最後に被験歯を観察した日までの期間

	中央値(日)	範囲(最小－最大)
プラセボ投与群(20名)	2,887	714－3,143
0.03%FGF－2投与群(19名)	2,879	263－3,093
0.1%FGF－2投与群(20名)	2,886	168－3,150
0.3%FGF－2投与群(20名)	2,868	249－3,122

3. イベント

各群で発生したイベントの内容と発生数を表2に示す。イベントが発生した14名の中で，積極的な介入をした非外科的歯周治療の発生数が7名と最も多かった。続いて，歯周組織再生療法（EMD，GTR等）が3名と多く，抜歯と歯周組織再生療法を除く歯周外科手術がそれぞれ2名であった。その他，歯周炎の進行が原因となって生じた事象は全ての群で発生しなかった。また，各群のイベントの発生数は，プラセボ群6名，0.03%群4名，0.1%群3名，0.3%群1名であった（図1および表2）。

4. 生存時間解析

治験薬投与日（0日目）からの経過日数に対するイベント未発生率を表すKaplan-Meier曲線を図2に示す。生存時間解析の結果，0.3% FGF-2投与群はプラセボ群に比べてイベント発生までの期間の有意な延長が認められた(一般化Wilcoxon検定：$p=0.0345$)。すなわち，0.3%FGF-2投与群では投与後2800日(約7.6年)程度で1名にイベントが発生し，観察期間の最終的なイベント未発生率は約80%であった。これに対してプラセボ群では，投与後2000日(約5.4年)まではイベント未発生率80%を維持していたものの，それ以降にイベント発生が増加し，最終的なイベント未発生率は約60%であった（図2）。

5. 安全性

本研究の観察期間中に，被験歯に異常な歯周組織の治癒（歯肉増殖等）が疑われる所見は認められなかった。

表2　発生したイベントと各群における発生数

(1)抜歯	全体 2名 (2.5%)
(治験薬投与部位を有する歯根の抜去を含む)	プラセボ: 1名
	0.03%: 0名
	0.1%: 1名
	0.3%: 0名
(2)歯周組織再生療法	全体 3名 (3.8%)
(EMD, GTR等)	プラセボ: 2名
	0.03%: 1名
	0.1%: 0名
	0.3%: 0名
(3)歯周組織再生療法を除く歯周外科手術	全体 2名 (2.5%)
	プラセボ: 0名
	0.03%: 0名
	0.1%: 2名
	0.3%: 0名
(4)積極的な介入をした非外科的歯周治療	全体 7名 (8.9%)
(歯肉縁下の処置を目的としたスケーリング・ルートプレーニング, 局所抗菌薬投与等)	プラセボ: 3名
	0.03%: 3名
	0.1%: 0名
	0.3%: 1名
(5)その他, 歯周炎の進行が原因となって生じた事象(逆行性歯髄炎等)	全体 0名 (0%)
	プラセボ: 0名
	0.03%: 0名
	0.1%: 0名
	0.3%: 0名
合計	全体14名 (17.7%)
	プラセボ: 6名
	0.03%: 4名
	0.1%: 3名
	0.3%: 1名

考　察

現在，進行した歯周炎に対する歯周外科処置としては，組織付着療法に分類されるフラップ手術が標準的治療法として施行されている。フラップ手術は，歯根面および歯周ポケット内部に蓄積した細菌および細菌由来病原物質を汚染セメント質とともに除去することが可能であり，術後には，炎症の軽減，臨床的アタッチメントの獲得，歯周ポケットの減少が認められる[7]。しかしながら，フラップ手術単独では，本来の歯周組織に見られる歯槽骨，歯根膜およびセメント質の新生を伴う線維性付着はわずかしか形成されず，歯周組織の再生はほとんど期待できない。また，臨床的アタッチメントの獲得も長い上皮性付着の形成によることが多く[8]，長期的には再度の付着喪失を伴う歯周炎の再発が懸念される。そのため，フラップ手術等の従来の歯周外科処置では達成できない歯周組織の再生に対する臨床現場の期待は非常に大きく，盛んに研究開発が行われてきた。そして現在，歯周組織再生誘導法（GTR法）およびエナメルマトリックスタンパク（EMD）を用いた歯周組織再生療法が我国で臨床適応されており，米国では血小板由来増殖因子（PDGF-BB）と骨補填材である多孔性リン酸三カルシウム（β-tricalcium phosphate; β-TCP）との合剤がヒト型リコンビナントサイトカインを用いた歯周組織誘導材料として登場している[9]。しかしながら，これらの方法は，治療成績が術者の技術レベルに大きく左右されたり，動物由来製剤であったりといった問題点から，重度歯周炎に対する標準的治療法となり得る歯周組織再生誘導薬の開発が強く求められている。そこで，我々は，様々な細胞に対する生理活性を有するFGF-2を，歯周組織再生誘導薬の有力な候補の一つと考え，その歯周組織誘導能の動物実験[2-4]による確認を経て，3，2壁性歯槽骨欠損を有する歯周炎患者を対象とした第II相臨床治験[5,6]を展開してきた。その結果，FGF-2投与36週後において，通常のフラップ手術と同程度の付着を獲得するとともに有意な歯槽骨新生がもたらされたことから，FGF-2の歯周組織再生誘導薬としての有用性が強く示唆されている。そこで，本研究では，歯周組織再生療法の真の目的が長期的な歯の保存であることを考慮し，投与36週後において歯周組織再生誘導薬としての有用性が確認されたFGF-2のさらに長期的な効果と安全性を検討するため，第II相臨床治験の中でも臨床治験終了後の経過期間が約8年と長期に及ぶKCB-1D探索的試験[5]に参加した被験者を対象として，フラップ手術時に各種濃度のFGF-2あるいはプラセボを投与した歯の長期経過を調査した。

歯周組織の再生を歯槽骨，セメント質，歯根膜などの再生と定義[10]すれば，これらの組織の再生を直接かつ明確に確認する唯一の方法は組織学的評価である。しかしながら，ヒトでの組織学的な評価は倫理的な観点から困難を極めるため，一般的に臨床試験では，規格エックス線写真による歯槽骨レベルの評価や臨床的アタッチメントレベル（CAL）の測定が歯周組織再生を評価する代替評価項目として利用される[10]。しかしながら，数年を超える長期の臨床経過の追跡では，その間に咬耗・摩耗や修復処置による歯冠形態の変化や歯の移動が生じるケースが多く，X線検査の規格化や一定の基準点を設定したCALの測定は，実際には不可能な場合が多い。そこで，本研究では，一連の動的な歯周治療を受けサポーティブペリオドンタルセラピィを受けている被験者では，歯周炎の治療経過が悪

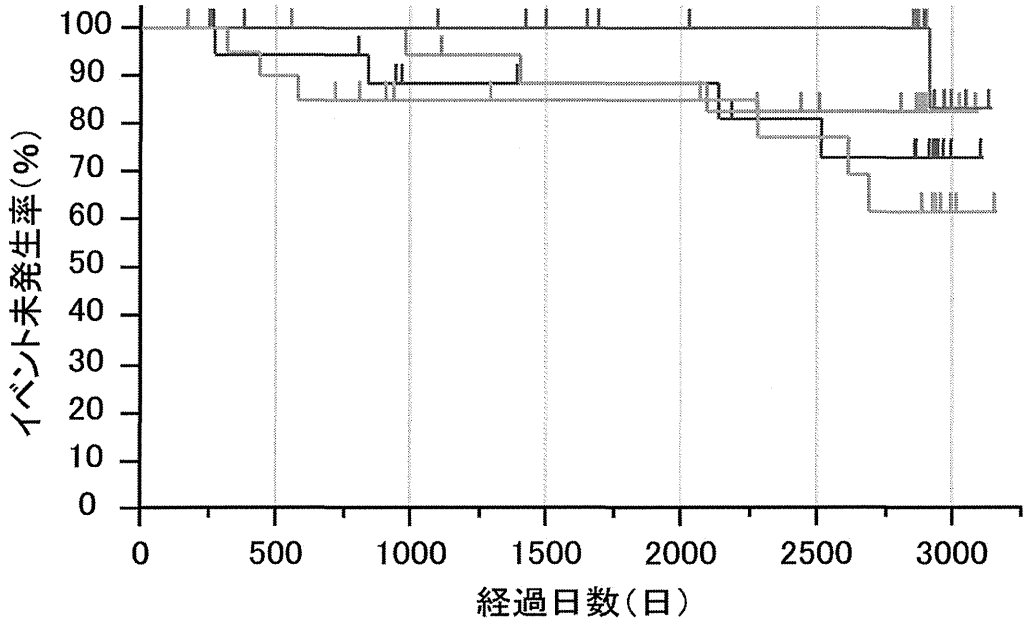

図2 イベント未発生率を表すKaplan-Meier曲線

赤：0.3%FGF-2投与群，緑：0.1%FGF-2投与群，青：0.03%FGF-2投与群，桃：プラセボ投与群。各群のKaplan-Meier曲線上のヒゲは打ち切りを表す。生存時間解析の結果，0.3%FGF-2投与群はプラセボ群に比べてイベント発生までの期間の有意な延長が認められた（一般化Wilcoxon検定：$p=0.0345$）。

く抜歯に至るケースでも抜歯に至る前に歯周外科的処置や歯周ポケットに対する非外科的な処置が通常行われることにヒントを得て，表2に示した(1) 抜歯，(2) 歯周組織再生療法，(3) 歯周組織再生療法を除く歯周外科手術，(4) 積極的な介入をした非外科的歯周治療 (5) その他，歯周炎の進行が原因となって生じた事象をイベントとして，FGF-2投与の長期的効果を判定する評価指標とした。

本研究では，転院等によって診療情報の一部が得らなかったため観察期間に治療等が発生したか否かを十分判断できなかった被験者は全被験者（79名）の約30%（25名）であった（図1）。しかしながら，KCB-1D探索的試験[5]で割り付けられた被験者の背景情報および調査途中打切り被験者数に大きな群間の偏りがなく，本研究における各群の観察期間が各群で同程度であったことから（表1），各群の被験者の一般的特性が結果に大きな影響を与えることはなかったと考えられる。

観察期間における各群のイベントの発生数は，プラセボ群6名，0.03%群4名，0.1%群3名，0.3%群1名と，高濃度のFGF-2を投与した群ほど低かった（図1および表2）。さらに，生存時間解析の結果においても，0.3% FGF-2投与群がフラップ手術単独施行に相当するプラセボ群に対して有意にイベント発生までの時間を延長させることが示され，0.3%FGF-2投与が，歯周炎の再発・悪化のリスクを長期的に低減することが示唆された（図2）。

本研究で被験歯に歯周炎の悪化による抜歯を認めたのは約2.5%(2歯)で，当初予想した通り非常に少なかった。Palcanis[11]はフラップ手術を含む歯周外科処置が歯の長期的な保存に与える影響を総説として報告している。この総説によると，Ramfjordら[12]は，歯周外科処置を行った1800歯の5年間の経過観察によって22歯(約1.2%)が抜去されていたことを報告しているが，その他の多くの長期観察では抜歯はほとんど認められていない。また，5年未満の観察では，フラップ手術後の臨床的アタッチメントの喪失は多くの場合1mm未満であり，5年程度の経過観察ではフラップ手術とFGF-2投与群との術後のイベント発生に大きな違いを認めなかったという本研究の結果と合致する。今回対象とした被験者が参加したKCB-1D探索的試験[5]の結果を受け2005年～2007年に実施されたKCB-1D用量反応試験[6]における同様の観察では，観察期間が約5年間と本研究の観察期間の8年より短く，イベントの発生に統計学的に有意な群間差を認めていない（結果未発表）。以上の結果から，治療に

よる長期的予後の違いを検討するには本研究と同程度か，それ以上の観察期間を要するものと考えられる。今後，より多くの被験者が参加した臨床治験を対象とした同様の観察研究の検討が望まれる。

本研究の結果も含め，これまでの研究からフラップ手術後に認められる長い上皮性の付着による治癒形態は，術後5年程度であれば維持されることが示唆されることから，フラップ手術が患者に与えるメリットは少なくない。しかしながら，それ以降の長期的な歯の予後には，FGF-2投与により歯槽骨の再生および結合性付着の再構築を図ることが有益であることが本研究で示された。世界初の歯周組織再生薬として市場に出たFGF-2が，歯周炎患者の歯の喪失を防ぎ，口腔の働きが支えるQOLの向上に寄与することが期待される。

結　論

FGF-2を用いた歯周組織再生療法臨床治験の施行後約8年間の観察で，0.3% FGF-2投与がフラップ手術単独と比較して再治療等のイベント発生までの期間を延長させることが示された。また，フラップ手術単独群のイベントの発生率は5年以降に増加する傾向が認められ，治療による長期的予後の違いを検討するには本研究と同程度かそれ以上の観察期間を要することが示唆された。また，本研究の観察期間を含めて安全性に関する問題は認めなかった。

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歯の保存に対するSupportive Periodontal Therapyの長期的効果

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抄録

目的：本研究では，supportive periodontal therapy（SPT）の歯の保存に対する有効性を検討した．

方法：約3カ月ごとのリコール間隔で10年以上SPTを受けている268人の歯周炎患者の残存歯数と歯の喪失を調査し，その推移を平成17年歯科疾患実態調査と比較した．

結果：被験者は動的歯周治療が終了したSPT開始時（平均年齢50.8歳）に平均24.4本の歯を有し，その後10年間のSPT期間に年平均0.22本の歯を喪失したが，その喪失歯の割合は上記実態調査の同年齢層の人よりも少なかった．さらに，SPT開始時の残存歯数が同実態調査で示された同年齢層の残存歯数より少なかった被験者（105人）も，平均16.7年間のSPT後には同調査における同年齢層の人よりも多数の歯を保有していた．

結論：SPTが歯の保存に効果的であることが明らかとなった．

キーワード：Supportive periodontal therapy，残存歯，歯周炎患者

RESEARCH ARTICLE

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CDH13 Gene Coding T-Cadherin Influences Variations in Plasma Adiponectin Levels in the Japanese Population

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ABSTRACT: Adiponectin is most abundantly expressed in adipose tissue and well known to play an important role in metabolic regulation. Several studies have attempted to identify the genetic determinants of metabolic syndrome (MetS), though no study has revealed a *cis-* or *trans-*single nucleotide polymorphism (SNP) that affects plasma adiponectin levels, except the adiponectin structure gene and genes encoding adiponectin-regulatory proteins. We performed a genome-wide association study in regards to plasma adiponectin concentrations in 3,310 Japanese subjects. We identified the strongest statistically associated SNP (rs4783244) with adiponectin levels ($P = 3.8 \times 10^{-19}$) in the first intron of *CDH13* (T-cadherin) gene in a 30-kb haplotype block covering the promoter region to first intron. In addition, rs12051272 SNP genotypes in linkage disequilibrium with rs4783244 were found to be more significantly associated with adiponectin levels ($P = 9.5 \times 10^{-20}$) and specifically with the levels of high-molecular weight (HMW) adiponectin, a subtype form associated with parameters related to glucose metabolism. Our results did show more significant association with adiponectin levels than rs12444338 (in *CDH13*) SNP genotypes reported recently. We suggest that the phenotype-affecting haplotype tagged by rs12051272 SNP would affect the plasma adiponectin levels and that we have to take the CDH13 genotype into account before considering the functional relevance of the adiponectin level.
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KEY WORDS: GWAS; CDH13; adiponectin; SNP

Introduction

Adiponectin is one of the most abundant gene products expressed in adipose tissue [Maeda et al., 1996]. It is also well known to play an important role in metabolic regulation affecting obesity, insulin sensitivity, or atherosclerosis [Yamauchi et al., 2001]. Plasma adiponectin levels are known to be correlated with body mass index (BMI), type 2 diabetes mellitus (T2DM), or even coronary artery disease [Hotta et al., 2000]. Several studies have also shown that adiponectin plays many metabolic effects including antidiabetic, antiatherosclerotic, or anti-inflammatory action [Matsuzawa et al., 2004]. Also, it is suggested to affect the relationship between obesity and insulin resistance or T2DM [Kern et al., 2003]. Production of adiponectin and plasma adiponectin concentrations was known to be regulated by complexed mechanisms [Yu and Ginsberg, 2005]. For example, its expression is increased by leanness, adrenalectomy, insulin-like growth factor 1, ionomycin, or thiazolidinediones, while it is decreased by obesity, tumor necrosis factor-α, glucocorticoids, β-adrenergic agonists, or cyclic AMP. In addition, genetic factors are also suggested to regulate adiponectin concentration as shown by the family study [Menzaghi et al., 2007] or several genome-wide linkage scans [Yang and Chuang, 2006]. Several candidate genes include the adiponectin structural gene (*ADIPOQ*; MIM# 605441) as well as the genes encoding adiponectin–regulatory proteins have been postulated to influence the adiponectin concentration [Ntalla et al., 2009], though the role of genetic variants regulating adiponectin function on insulin resistance, T2DM, or coronary artery disease, has not been clearly determined.

Recently, the development of low-cost, high-throughput genotyping technology made it possible to identify common genetic variants influencing health outcomes on a genome-wide scale. Several studies were performed to identify the genetic determinants of metabolic syndrome (MetS) and related traits. In one study, a comprehensive assessment of the genetic determinants of adiponectin levels was performed on a genome-wide basis in northern and western European population in addition to genome-wide linkage and association analyses, and the genetic influences on plasma levels of adiponectin were evaluated [Ling et al., 2009].

Here, to identify genes influencing variation in plasma adiponectin levels, we performed a genome-wide association study on plasma adiponectin concentration in subjects recruited in Suita, Osaka, Japan.

Materials and Methods

Suita Study

The Suita study was initiated as a cohort study for cardiovascular diseases of urban residents of Japanese in 1989. The details of this study were described elsewhere [Iwai et al., 2002]. Data from 5,098

Additional Supporting Information may be found in the online version of this article.

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participants (2,404 men and 2,694 women) were initially included in the analysis. DNA samples were prepared from 3,310 participants (1,527 men and 1,783 women) after informed consent was obtained. This cohort study was approved by the ethics committee of the National Cardiovascular Center. Subjects received a physical exam during which height, weight, and waist circumference were measured according to a standardized protocol. Blood samples were collected after a 12-hr fast. Plasma adiponectin level and other blood chemical levels including serum total cholesterol (TC), high density lipoprotein (HDL) cholesterol, triglyceride (TG), glucose, and insulin were measured. Hypertension was defined as either a systolic blood pressure (SBP) ≥140 mm Hg, a diastolic blood pressure (DBP) ≥90 mm Hg, or the use of antihypertensive agents. Diabetes was defined as a fasting serum glucose ≥7.0 mmol/L (126 mg/dl), the use of antidiabetic agents, or both. MetS was defined using modified NCEP-ATP III criteria [Heng et al., 2006] based on the International Obesity Task Force central obesity criteria for Asia [Kanazawa et al., 2002].

Yahaba Study

The Yahaba study was initiated as a cohort study for cardiovascular diseases of rural residents in 2007. DNA samples were prepared from 172 participants (69 men and 103 women) after informed consent was obtained. This cohort study was also approved by the ethics committee of the National Cardiovascular Center. Blood samples were collected as the Suita study. The samples were also used for the measurement of the same parameters performed in the Suita study.

Adiponectin Measurement

Plasma adiponectin level was measured using an enzyme-linked immunosorbent assay (Mercodia, Uppsala, Sweden). Prior to genetic analyses, mean levels of adiponectin and other traits were compared between groups using linear regression in SAS version 9 (SAS Institute, Cary, NC).

Association Study

The association study was initially carried out to identify specific single nucleotide polymorphisms (SNPs) associated with variation in log-transformed adiponectin levels in the Suita Study. Genotyping was initially performed using the Illumina 550K chip (Illumina, San Diego, CA). TaqMan system (Life Technologies, Carlsbad, CA) was then used for further genotyping of the individual SNPs. Exclusion criteria of SNP genotypes for this study were minor allele frequency (MAF) <0.1, *P*(Hardy–Weinberg equilibrium) <0.05, or typing rate <0.98. The genome-wide SNP association analysis for adiponectin was performed in a simple linear regression and additive genetic model without adjustment. In addition to reporting marker-wise statistical test results, genome-wide levels of statistical significance were calculated by applying a Bonferroni correction. Linkage disequilibrium structure was evaluated at selected locus regions using the Haploview software (Broad Institute, Cambridge, MA). For further genotyped individual SNPs, association analysis was performed in a multiple linear regression model with adjustment for age, sex, and BMI.

Results

The statistical analysis in the initial stage (Suita-1) was performed with the data from 842 individuals of Suita study. Based on the exclusion criteria of SNP genotypes, 348,622 SNPs were analyzed. Figure 1 shows genome-wide association with plasma adiponectin as well as a plot of the *P*-values of each SNP according to its physical location encompassing *CDH13* gene (MIM# 601364) region in the initial screening of samples of Suita study. In this analysis, genomic inflation factor (based on median chi-squared) was 1 and cutoff *P*-value after applying a Bonferroni correction was 1.43×10^{-7}. Seven SNPs (four SNPs in choromosome 11 and three SNPs in chromosome 16) were significantly associated with plasma adiponectin. Since these SNPs in chromosome 16 (rs4783244:G>T, rs9940180:C>T, rs7193788:A>G) were located in *CDH13* gene but SNPs in chromosome 11 (rs563272:C>T at 115512749, rs483058:A>G at 115513725, rs7125373:A>G at 115532483, rs1621764:C>T at 115538977) were not located within or near the known genes, we concentrated SNPs located in chromosome 16 in this study. We also evaluated the linkage disequilibrium plots of these SNPs in chromosome 16. The strongest statistically associated SNP (rs4783244) lay in the first intron of *CDH13* gene in a 30-kb haplotype block covering the promoter region to first intron. Haplotype analysis using seven SNPs in this block (rs16957844:A>C, rs3844412:C>T, rs3865186:C>T, rs9940180, rs7193788, rs4783244, rs8047711:A>G) revealed that the haplotype with the strong evidence of association was indeed tagged by a single tag SNP, rs4783244 (Supp. Table S1). Since the selection of tag SNPs in Illumina Infinium II assay was based on the haplotype data of Caucasian population, we next evaluated the haplotype block structure in Japanese population using genotype data available from the international HapMap project. Although the extent of haplotype block of this region in Japanese was similar with that in Caucasians based on the calculated linkage disequilibrium (LD) measure r^2 (Supp. Fig. S1), major haplotype structure consisting the haplotype block including rs4783244 was quite different, suggesting we should reassess the tag SNP selection using Japanese set of SNP data. All HapMap SNPs available in 33-kb region bounded by SNPs rs2318177:C>T and rs8045889:C>T that covers the 30-kb haplotype block, were screened by LD measure—presenting the $r^2 < 0.7$ with all tag SNPs in Illumina assay system. Seven SNPs (rs11646213:A>T, rs12051272:G>T, rs16957848:C>T, rs3852729:A>C, rs3865183:A>G, rs6565051:A>G, rs8060461:A>G) met these criteria. Of these, six SNPs (rs11646213, rs16957848, rs3852729, rs3865183, rs6565051, rs8060461) were excluded since they showed stronger r^2 with other SNPs presented in Illumina assay system than with rs4783244 (Supp. Table S2). So, the TaqMan probe set predesigned for the remained rs12051272, which had been excluded from Illumina assay system because of a low MAF in Caucasian population (Supp. Table S2), was obtained and genotyped in the same Japanese population. Interestingly, the rs12051272 SNP presented even stronger association with plasma adiponectin levels than rs4783244 ($P = 2.6 \times 10^{-13}$ for unadjusted, and 9.5×10^{-20} adjusted for sex, age, and BMI) in this initial stage study (Suita-1).

Next we genotyped these two SNPs, rs4783244 and rs12051272, in the whole set of Suita Study subjects using a TaqMan PCR method (Table 1). We intensively included the samples from the first screening to measure the typing discrepancy between two methods. The replicate error rate between two methods in rs4783244 was 0.59%. Those five subjects with ambiguous data were accordingly excluded from the further analysis.

The statistical analysis in the second stage (Suita-2) was then conducted with the data from remaining 2,468 Suita study participants left out from the first screening (Table 1). The basic characteristics of these two groups are similar as shown in Table 2. The mean age was older in the second group as expected since the aged participants were excluded from the first screening. Accordingly, the mean adiponectin level, which was known to be affected by age, was higher

Table 1. Results of Genotyping Two SNPs, rs4783244 and rs12051272, in the *CDH13* Gene, in Suita-1, Suita-2, or Yahaba

Set				Suita-1					Suita-2					Yahaba				
Number(M/F)				373/464					1144/1311					68/101				
						P-value					*P*-value					*P*-value		
SNP	MAF			Mean	SD	Unadj	a,s adj	a,s,B adj	Mean	SD	Unadj	a,s adj	a,s,B adj	Mean	SD	Unadj	a,s adj	a,s,B adj
rs4783244	0.31	Female	11	6.26	4.96	3.56×10^{-8}	1.51×10^{-8}	8.98×10^{-9}	8.00	5.57	2.26×10^{-9}	2.09×10^{-8}	2.35×10^{-10}	9.53	4.60	0.32	0.33	0.30
			12	8.06	5.42				8.60	5.71				11.4	6.95			
			22	9.78	6.49				10.0	6.38				11.8	8.82			
		Male	11	3.60	1.57	5.17×10^{-9}	1.63×10^{-9}	1.11×10^{-12}	4.72	3.62	8.14×10^{-10}	4.85×10^{-11}	6.30×10^{-13}	4.91	5.22	5.88×10^{-5}	6.33×10^{-5}	7.32×10^{-5}
			12	5.12	3.23				5.79	4.54				6.03	5.29			
			22	6.12	4.34				6.64	4.98				10.0	4.58			
		Combined	11	4.80	3.60	7.87×10^{-13}	1.55×10^{-16}	3.83×10^{-19}	6.30	5.25	4.24×10^{-15}	2.31×10^{-17}	1.60×10^{-21}	7.31	6.33	0.0031	0.0007	0.0006
			12	6.65	4.89				7.15	5.58				9.06	7.69			
			22	7.87	6.06				8.26	6.25				11.0	6.96			
rs12051272	0.31	Female	11	5.83	4.28	3.82×10^{-9}	1.63×10^{-9}	1.45×10^{-9}	7.94	5.55	1.53×10^{-9}	1.18×10^{-8}	1.49×10^{-10}	9.53	4.60	0.45	0.45	0.42
			12	8.12	5.40				8.58	5.78				11.6	6.92			
			22	9.70	6.60				10.0	6.33				11.6	8.78			
		Male	11	3.65	1.65	8.81×10^{-9}	3.22×10^{-9}	2.30×10^{-12}	4.68	3.62	7.60×10^{-10}	4.39×10^{-11}	5.32×10^{-13}	4.91	5.22	5.88×10^{-5}	6.33×10^{-5}	7.32×10^{-5}
			12	5.08	3.23				5.78	4.53				6.03	5.29			
			22	6.11	4.31				6.63	4.95				10.0	4.58			
		Combined	11	4.65	3.23	2.61×10^{-13}	2.82×10^{-17}	9.46×10^{-20}	6.25	5.24	2.61×10^{-15}	1.03×10^{-17}	8.07×10^{-22}	7.31	6.33	0.0047	0.0013	0.0011
			12	6.68	4.93				7.14	5.61				9.12	7.77			
			22	7.85	6.08				8.25	6.21				10.9	6.96			

M, male; F, female; MAF, minor allele frequency; 1, minor allele; 2, major allele; unadj, unadjusted; a,s adj, age and sex adjusted; a,s,B adj, age, sex, and BMI adjusted.

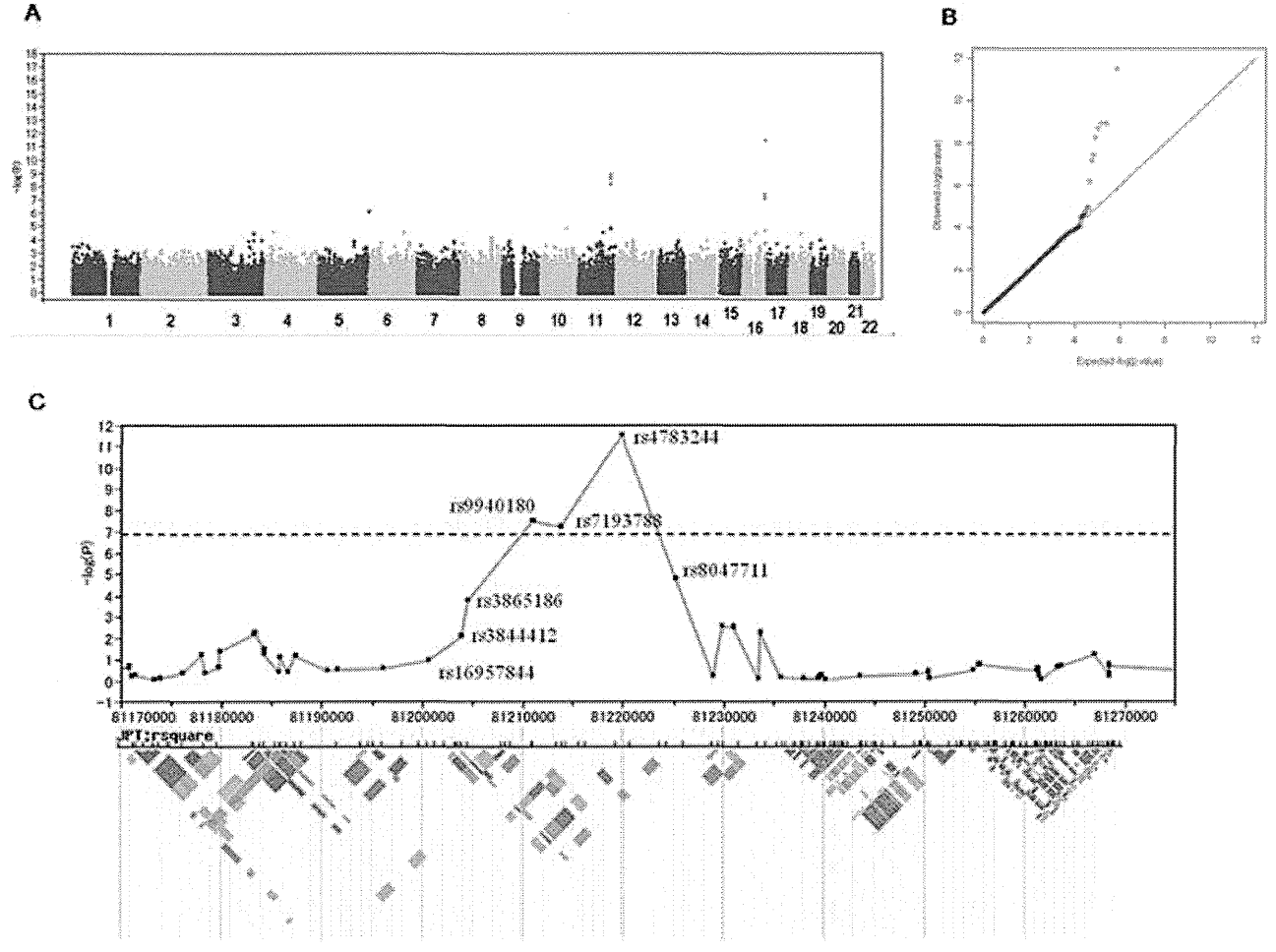

Figure 1. Genome-wide association with plasma adiponectin in Suita population. **A:** Manhattan plot. **B:** PP plot. **C:** *P*-values of each SNPs according to its physical location encompassing *CDH13* gene region were shown.

in the second group ($P < 0.001$). But the intergroup difference in plasma adiponectin levels was not significant after adjustment for age and sex ($P = 0.23$). Using the second group, the two SNPs examined showed again statistically significant association with log-transformed plasma adiponectin levels ($P = 4.2 \times 10^{-15}$ and 2.6×10^{-15} for rs4783244 and rs12051272, respectively). The association was even more significant after adjustment for sex, age, and BMI ($P = 1.6 \times 10^{-21}$ and 8.1×10^{-22}). The association of rs12051272 alleles was slightly more significant than that of rs4783244, same as the result for the first group ($P = 3.8 \times 10^{-19}$ and 9.5×10^{-20}, respectively after adjustment for sex, age, and BMI). The evidence of association was strongest ($P = 4.4 \times 10^{-37}$ and 6.9×10^{-38}) when data from two groups were combined (Suita-1 + Suita-2) and adjusted for sex, age, and BMI (Supp. Table S3). Finally, pairwise LD (r^2) between rs4783244 and rs12051272 in Suita study group was 0.97. From these data, we concluded that the haplotype tagged by rs12051272 was most responsible for the association with plasma adiponectin levels.

Table 2. The Basic Characteristics of Groups for the Study

	Suita-1	Suita-2	Yahaba	
Number	842	2468	172	
Female sex percent	55.6	53.3	59.9	
Age	58.2 (7.0)	66.7 (11.3)	62.2 (6.6)	b,c
BMI (kg/m^2)	22.8 (2.9)	22.9 (3.2)	24.3 (3.1)	c
Waist/hip ratio	0.884 (0.046)	0.907 (0.052)	0.913 (0.073)	b,c
Adiponectin (μg/dl)	7.0 (5.5)	7.5 (5.9)	9.8 (7.6)	b,c
TC (mg/dl)[a]	211.5 (32.5)	207.4 (32.3)	211.0 (32.8)	b
LDLc (mg/dl)	128.4 (31.1)	126.1 (29.5)	131.6 (32.0)	
HDLc (mg/dl)	61.5 (15.6)	59.9 (15.6)	60.3 (14.0)	b
TG (mg/dl)	92.7 (65.7)	93.4 (61.6)	100.9 (58.9)	c
SBP (mm Hg)[a]	123.1 (17.3)	131.4 (19.6)	122.8 (20.1)	b,c
DBP (mm Hg)	77.4 (10.0)	78.0 (10.0)	73.0 (12.0)	c
Fasting insulin (μU/ml)	4.4 (3.3)	4.8 (3.8)	5.5 (4.9)	b,c
Fasting glucose (mg/dl)[a]	94.8 (14.4)	99.2 (18.8)	88.1 (14.2)	b,c

All measures were shown in mean (SD).
[a]Variables were log tranformed for initial calculation of mean and SD. Values in table were shown as untransformed values.
[b]Statistically significant difference.
[c]Statistically significant difference between Suita (1 + 2) and Yahaba cohort.

Since the first and second groups were derived from the single cohort, though they did not overlap each other, we next conducted an additional analysis using the data from Yahaba study, another residential cohort study originated in northern part of Japan to confirm our results from Suita study. The population characteristics of these two studies were different in several points. The cohort in Suita study are residents in the urban area of Osaka, the second largest city of Japan, and most of the residents originated in western part of Japan, and settled down about half a century ago. In contrast, Yahaba is a small town located in the rural area of northern part of Japan and its

Table 3. SNP Genotypes in CDH13 and Adiponectin Types in Yahaba

		HMW adiponectin						Non-HMW adiponectin				
						Additive *P*-value					Additive *P*-value	
SNP	MAF		Genotype	Mean	SD	Unadj	a,s adj	a,s,B adj	Mean	SD	Unadj	a,s adj	a,s,B adj
rs4783244	0.30	Female	22	7.43	6.96	0.46	0.46	0.42	4.67	2.19	0.15	0.15	0.13
			12	7.36	5.15				4.26	1.84			
			11	6.00	3.15				3.72	1.45			
		Male	22	6.38	3.48	4.10×10^{-5}	4.50×10^{-5}	3.80×10^{-5}	3.69	1.38	0.24	0.23	0.38
			12	3.04	3.39				3.31	1.77			
			11	3.33	2.36				3.05	1.10			
		Combined	22	6.95	5.40	0.0071	0.001	0.001	4.24	1.93	0.113	0.062	0.056
			12	5.35	5.94				3.92	1.86			
			11	4.86	3.42				3.48	1.34			
rs12051272	0.30	Female	22	7.33	6.86	0.56	0.56	0.52	4.61	2.21	0.22	0.22	0.20
			12	7.46	5.19				4.32	1.82			
			11	6.00	3.15				3.72	1.45			
		Male	22	6.38	3.48	4.13×10^{-5}	4.50×10^{-5}	3.83×10^{-5}	3.69	1.38	0.24	0.23	0.38
			12	3.04	3.39				3.31	1.77			
			11	3.33	2.36				3.05	1.10			
		Combined	22	6.91	5.36	0.009	0.002	0.002	4.21	1.94	0.156	0.095	0.089
			12	5.37	6.02				3.95	1.85			
			11	4.86	3.42				3.48	1.34			

MAF, minor allele frequency; 1, minor allele; 2, major allele; unadj, unadjusted; a,sadj, age and sex adjusted; a,s,B adj, age, sex, and BMI adjusted.

residents were mostly indigenous since 1800s. The rs4783244 and rs12051272 SNPs were genotyped using the TaqMan PCR method as described earlier. Although the sample size in Yahaba was smaller compared to the previous two studies, we found a statistically significant association ($P = 0.0006$ and 0.0011, respectively) after male and female were combined. Here, female samples in Yahaba did not show significant association between adiponectin levels and either SNP genotype under any genetic model including a dominant model (data not shown), while the MAF in Yahaba was 0.31 and not different from that for Suita study. This might be due to small number of Yahaba study or characteristics of Yahaba female. *CDH13* gene codes for T-cadherin that had been identified as a receptor specific for high-molecular weight (HMW) adiponectin [Hug et al., 2004]. We next examined if the association was specific for the levels of HMW adiponectin and not for that of low-molecular weight (LMW) adiponectin using data from Yahaba study. As expected, rs12051272 SNP genotypes were significantly associated only with the levels of HMW adiponectin ($P = 0.0018$ after adjustment for sex, age, and BMI) and not with that of other types of adiponectin ($P = 0.09$) (Table 3). However, there was weaker association between SNP genotype and HMW adiponectin in female than in male partly because of sex difference in plasma adiponectin concentrations (female: 11.4 μg/dl, male: 7.7 μg/dl, $P = 9 \times 10^{-6}$). Since several SNPs or mutant within the *ADIPOQ* gene, which codes for adiponectin, had been reported to be associated with plasma adiponectin levels previously, we genotyped these SNPs additionally. These included rs2241766:G>T (Gly15:exon1), rs1501299:A>C (intron1), rs710445:A>G (promoter), and Ile164Thr (nonsynonymous substitution). They indeed showed a weak but statistically significant association with adiponectin levels in our Suita study subjects ($P = 0.00093, 0.32, 0.0001, 2.2 \times 10^{-25}$, respectively), but the magnitude of significance was smaller compared that of *CDH13* SNP rs12051272. The effect size presented in eta-squared for rs12051272, rs2241766, rs1501299, rs710445, and Ile164Thr was 3.28%, 0.27%, 0.02%, 0.37%, and 2.62%, respectively. The beta-coefficient per allele for them was 27.7%, 8.0%, 2.4%, 8.7%, and 127.5%, respectively. Ile164Thr mutant showed the largest beta-coefficient, but the MAF was very low (0.8%), resulting in smaller eta value than that of rs12051272 (Table 4).

Since T-cadherin has been shown to bind low density lipoprotein (LDL) cholesterol [Resink et al., 1999], we next analyzed the association between rs12051272 genotypes and the levels of LDL cholesterol using the data from whole Suita study participants. The rs12051272 genotypes showed no significant association with serum LDL cholesterol levels ($P = 0.19$), as well as with total or HDL cholesterol levels ($P = 0.46$ and 0.16, respectively). Plasma adiponectin levels also have been shown independently related with many obesity-related phenotypes, including insulin resistance [Lindsay et al., 2002; Snehalatha et al., 2003], the levels of fasting insulin, fasting glucose, and fasting TG. Since they showed a significant association also in our study, we next evaluated the association between those phenotypes and the rs12051272 genotypes (Table 5A). Interestingly, although we failed to find any statistical association between them in simple regression analysis adjusted for age and sex, we found a significant association when log-transformed plasma adiponectin level was included as an explanatory variable. The partial correlation coefficient between plasma adiponectin levels and homeostasis model assessment-estimated insulin resistance (HOMA-IR), the levels of fasting insulin, fasting glucose, and fasting TG in Suita study population was −0.39 ($P = 5.00 \times 10^{-102}$), −0.38 ($P = 7.04 \times 10^{-94}$), −0.23 ($P = 1.77 \times 10^{-34}$), and −0.36 ($P = 4.48 \times 10^{-85}$), respectively, while that of plasma adiponectin levels and rs12051272 genotypes (additive model) was −0.24 ($P = 2.0 \times 10^{-34}$). Similarly, that of

Table 4. Effect Size of SNPs on Serum Adiponectin Levels

		Serum adiponectin (μg/ml), mean (SD)				Eta-squared			Beta coefficient		
	Allele1 f	Allele 1	Both	Allele2	*P*-value (a)	SNP (a)	Age	Sex	SNP (p al)	Age	Sex (p sex)
rs12051272	0.688	8.01 (0.10)	6.87 (0.09)	5.75 (0.17)	2.0×10^{-31}	3.28%	5.77%	15.35%	27.7%	24.2%	79.1%
rs4783244	0.684	8.03 (0.11)	6.88 (0.09)	5.84 (0.17)	1.7×10^{-30}	3.17%	5.71%	15.43%	27.1%	24.1%	79.3%
rs2241766	0.295	7.66 (0.24)	7.45 (0.11)	7.05 (0.09)	9.3×10^{-4}	0.27%	5.82%	15.30%	8.0%	24.3%	79.0%
rs1501299	0.277	7.18 (0.23)	7.2 (0.11)	7.33 (0.09)	0.32	0.02%	5.93%	15.14%	2.4%	24.5%	78.6%
rs266729	0.748	7.4 (0.09)	7.16 (0.11)	6.77 (0.24)	0.009	0.17%	5.84%	15.29%	6.6%	24.3%	79.0%
rs710445	0.412	7.72 (0.17)	7.33 (0.10)	6.97 (0.11)	1.0×10^{-4}	0.37%	5.84%	15.14%	8.7%	24.3%	78.6%
rs822395	0.063	5.02 (0.86)	7.01 (0.19)	7.31 (0.07)	0.029	0.12%	5.84%	15.23%	10.0%	24.3%	78.8%
APM1(I164T)	0.008	NA	3.5 (0.25)	7.35 (0.07)	2.2×10^{-25}	2.62%	5.95%	15.51%	127.5%	24.6%	79.5%
APM1(H241P)	0.006	NA	8.44 (0.71)	7.25 (0.07)	0.062	0.09%	5.89%	15.25%	26.1%	24.4%	78.8%

Age, sex, and BMI adjusted ($N = 3310$).
f, frequency; (a), additive; (p al), per allele; (p sex), per sex.

Table 5. Association of Obesity-Related Phenotypes, Adiponectin, and the rs12051272 Genotypes in Suita (1 + 2)

	P-value (response variable = each parameter)				Partial correlation coefficient		
	rs12 (a v)	rs12 (a v) pl adipo adj	pl adipo	pl adipo, rs12 (a v) adj	betw rs12 (a v) and each	betw rs12 (a v) and pl adipo	betw pl adipo and each
			A. Adjustment of age and sex				
Fasting insulin[a]	0.84	4.05×10^{-6}	9.09×10^{-89}	7.04×10^{-94}	−0.088	−0.22	−0.38
Fasting glucose[a]	0.66	2.33×10^{-3}	1.17×10^{-32}	1.77×10^{-34}	−0.058	−0.21	−0.23
HOMA-IR[a]	0.77	8.62×10^{-7}	1.78×10^{-96}	5.00×10^{-102}	−0.094	−0.22	−0.39
Fasting triglyceride[a]	0.89	1.74×10^{-5}	2.92×10^{-81}	4.48×10^{-85}	−0.082	−0.22	−0.36
BMI	0.09	3.53×10^{-7}	4.78×10^{-69}	1.55×10^{-75}	−0.097	−0.22	−0.31
			B. Adjustment of age, sex, and BMI				
Fasting insulin[a]	0.40	0.015	6.73×10^{-43}	1.77×10^{-44}	−0.047	−0.22	−0.26
Fasting glucose[a]	0.98	0.036	7.12×10^{-18}	1.03×10^{-18}	−0.040	−0.22	−0.17
HOMA-IR[a]	0.46	0.0053	3.16×10^{-49}	4.07×10^{-51}	−0.053	−0.23	−0.28
Fasting triglyceride[a]	0.73	0.00074	7.01×10^{-56}	3.78×10^{-58}	−0.065	−0.23	−0.30

[a]Calculated by using logarithm. rs12:rs12051271. a v, additive variable; pl adipo, plasma adiponectin; adj, adjustment; betw, between.

each metabolic phenotype and the rs12051272 genotypes (additive model) was −0.094 ($P = 8.62 \times 10^{-7}$), −0.088 ($P = 4.05 \times 10^{-6}$), −0.058 ($P = 2.33 \times 10^{-3}$), and −0.082 ($P = 1.74 \times 10^{-5}$), respectively. The results were consistent if BMI was included as an explanatory variable (Table 5B). This means the SNP genotype has a statistically significant negative effect on those phenotypes independent of plasma adiponectin levels, with rs12051272-G allele increasing these metabolic risks, but it is cancelled by its positive effect through increasing adiponectin levels simultaneously. The mechanism how the SNP genotype has effects on those phenotypes independent of plasma adiponectin levels remains to be elucidated.

From the other point of view, when we analyzed the effect of plasma adiponectin concentration on those phenotypes, the association became even stronger when adjusted for rs12051272 genotypes (Table 5A, B). That suggests when we use the plasma adiponectin levels as a marker for risk of cardiovascular events or MetS in the clinical practice, as proposed by several researchers, the SNP genotypes of *CDH13* gene have to be considered. Based on our results that SNPs for *CDH13* gene were strongly associated with HMW adiponictin, we tried to adjust HMW adiponectin instead of plasma adiponectin, but we only observed similar results between adjusting HMW adiponectin and plasma adiponectin levels (data not shown).

During preparation of this manuscript, a genome-wide association study in Korean population as well as in Filipino women reported that rs3865188:A>T in *CDH13* was indeed associated with adiponectin levels [Jee et al., 2010; Wu et al., 2010] as shown here. Also, it showed that G variant at rs12444338:G>T in linkage disequilibrium with rs3865188 had an increased promoter activity of *CDH13* gene in vitro [Jee et al., 2010]. However, our results did show that rs12051272 SNP genotypes were more significantly associated with adiponectin levels than rs12444338 SNP genotypes (Table 1 and Supp. Table S4), while rs12444338 SNP genotypes were in linkage disequilibrium with rs4783244 and rs12051272 (Supp. Table S5).

Discussion

Adiponectin is an adipokine, secreted specifically and abundantly by adipose tissues. It has attracted much attention because of its antidiabetic and antiatherosclerotic effects by sensitizing the body to insulin. It has been shown that adiponectin inhibits hepatic glucose production, enhances glucose uptake in muscle, increases fatty acid oxidation in both liver and muscle, and augments energy expenditure by enhancing uncoupling of ATP generation in mitochondria in vitro. Numerous studies have shown that plasma adiponectin concentration correlates negatively with fasting plasma insulin levels and insulin resistance measures. Lower plasma levels of adiponectin have been shown to be associated with obesity, type 2 diabetes, coronary artery disease, hyperlipidemia, hypertension, and the MetS. Some investigators suggest that it can be considered as a biomarker or a diagnostic marker to predict vulnerability for MetS [Martin et al., 2005; Santaniemi et al., 2006], cardiovascular events [Pischon et al., 2004], and in-stent restenosis after acute myocardial infarction [Kitta et al., 2008; Moldoveanu et al., 2008].

Adiponectin exists in three major oligomeric forms; a LMW trimer, a middle-molecular weight (MMW) hexamer, and HMW 12- to 18-mer. A small amount of globular form, possibly resulting from proteolytic cleavage, has also been described. Recently, HMW adiponectin has been especially attracting attention because the level of HMW adiponectin was reported to be more significantly associated with parameters related to glucose metabolism than other forms. The level of HMW adiponectin or the ratio of HMW to total adiponectin was shown to be more relevant to the prediction of insulin resistance [Lara-Castro et al., 2006].

T-cadherin (*CDH13*), which is expressed in endothelium and smooth muscle, has been identified as a receptor specific for HMW adiponectin [Hug et al., 2004]. The amino acid motif of T-cadherin is well conserved in higher eukaryote compared to that of E-cadherin, suggesting of some biological significance. It lacks a cytoplasmic domain and is anchored to the surface membrane via glycosyl phosphatidyl inositol (GPI) moiety, it is speculated that T-cadherin may act as a co-receptor along with other signaling molecules, but its physiological roles are largely unknown. T-cadherin has been shown to be more strongly expressed in regenerative endothelial cells and vascular smooth muscle cells in the region of atherosclerosis than in those of the normal artery, and the level of its expression is known to be correlated with the progression of atherosclerosis, implicating that it is playing some role in atherosclerotic changes.

We showed a single SNP rs12051272, tagging a corresponding haplotype constituting of rs12051272 and rs4783244, was significantly associated with the plasma adiponectin levels. The association of the rs12051272 SNP genotypes and plasma adiponectin levels was consistent across all studies. The effect size measured in eta squared in Suita study population suggested that the two variants rs12051272 in *CDH13* gene and Ile164Thr of *ADIPOQ* explained 3.28% and 2.62% of the variation in plasma adiponectin levels, respectively. Interaction between rs12051272 in the *CDH13* gene and +517T>C (Ile164Thr) within *ADIPOQ* gene was not statistically significant, though the combined effects of these two SNPs on plasma adiponectin concentrations were shown to be additive (data not shown). Although the rs12051272 showed the smaller

beta-coefficient than Ile164Thr, the contribution in the population was greater because of the higher MAF.

Previously, a similar GWAS study adiponectin was reported [Richards et al., 2009]. They used the same SNP platform, Illumina HumanHap550, as ours but failed to identify any *cis*- or *trans*-SNP of *CDH13* affecting the plasma adiponectin levels. Illumina HumanHap550 assay contains a probe for rs4783244 but not that for rs12051272. The haplotype structure data obtained from Human HapMap project showed that the MAF of rs4783244 was similarly high in both Caucasians and Japanese (0.46 and 0.32), while that of rs12051272 was much lower in Caucasians (0.01) than in Japanese (0.25). Based on our study results and the above, we estimated the power of detection for the SNP rs12051272 in Japanese as 0.992, while we did that in Caucasian as only 0.00002. Our result suggests that the haplotype constituting T allele of rs12051272 (G/T) and T allele of rs4783244 (G/T), tagged by a single rs12051272 SNP, has an effect in decreasing plasma adiponectin levels. Illumina HumanHap550 does not contain the probe for rs12051272, along with the too low MAF of this TT haplotype to be detected of significant association in Caucasian population that contains mostly GG and TG haplotypes, were the main reasons why GWAS in Caucasian population failed to identify the significant association. The difference of MAF may also partly explain the difference in the baseline plasma adiponectin levels between Japanese and American. Kadowaki et al. showed that the American men had been shown to have higher levels of adiponectin than the Japanese men despite higher levels of obesity [Kadowaki et al., 2006]. Since the majority of American had adiponectin-increasing rs12051272-G alleles, the mean plasma adiponectin levels can be apparently higher accordingly, but this does not mean the Americans are more resistant to risk phenotypes because this allele has an independent effect on augmenting insulin resistance as shown by the partial correlation coefficients. The mean difference in plasma adiponectin levels between GG and TT groups in Japanese was around 2 μg/ml consistently in both sexes in three independent groups, so the sole genotypic difference in T-cadherin gene may not explain the 6 μg/ml difference between Japanese and Americans.

Compared with the report of the genome-wide association study in Korean population as well as in Filipino women, our results did show that rs12051272 SNP genotypes were more significantly associated with adiponectin levels and especially with the levels of HMW adiponectin than rs12444338 SNP genotypes.

Our results provide two novel insights relating T-cadherin. First, in analyzing the physiological effects of plasma adiponectin, the analysis has to be adjusted for the SNP genotypes that would affect the 28% of SD changes in plasma adiponectin level per allele.

Several researchers have proposed to use the plasma adiponectin levels as a marker for risk of cardiovascular events or MetS in the clinical practice. For that purpose, the effect of the SNP genotypes, with the mean difference of 1 μg/ml per allele, on the levels of adiponectin is too large to ignore, especially in Japanese. Second, T-cadherin should have some unknown effect independent of adiponectin levels, since the same allele had an opposite effect judging from the results of partial correlation coefficient. The effect of the rs12051272 SNP also has to be elucidated. Since the SNP is located in the first intron of *CDH13* gene and the surrounding nucleotide sequence did not match the known transcription factor binding site or miRNA-targeted sequence, it is likely that the rs12051272 is a mere marker SNP tagging the phenotype-affecting haplotype present in 30-kb haplotype block covering from the promoter region to the first intron of CDH13. The idea that phenotype-affecting haplotype tagged by rs12051272 SNP would affect the baseline level of T-cadherin in the tissues is attractive, since the increasing amount of T-cadherin may capture the free adiponectin molecules in the plasma resulting in lowering plasma HMW adiponectin levels, simultaneously increasing the adiponectin signals in the peripheral vessel walls resulting in augmenting the effect of adiponectin per cell. Therefore, we may have to take the CHD13 genotypes as well as ADIPOQ genotypes into account if the plasma adiponectin levels is used as a marker for a risk of cardiovascular events or diabetes, though the attempt to compare the level of T-cadherin in peripheral vessel walls with the genotype will give us further evidence for this result.

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Brief Report

Prenatal complex congenital heart disease with Loeys–Dietz syndrome

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Abstract We report an infantile case of Loeys–Dietz syndrome prenatally diagnosed with congenital complex heart disease – double outlet right ventricle and interruption of the aortic arch. The patient also showed prominent dilatation of the main pulmonary artery. Emergency bilateral pulmonary artery banding was performed on the 9th day. However, on the 21st day, the patient died of massive bleeding due to rupture of the right pulmonary artery. Subsequently, a mutation of the TGFBR1 gene was detected. As cardiovascular lesions of Loeys–Dietz syndrome appear early and progress rapidly, the prognosis is generally poor. Patients require periodic examination and early intervention with medical therapy such as Losartan administration and surgical therapy. Early genetic screening is thought to be useful for the prediction of complications as well as vascular disease.

Keywords: Prenatal diagnosis; aneurysm; chromosomal anomaly; connective tissue disorder

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Loeys–Dietz syndrome is a newly recognised, rare autosomal dominantly inherited connective tissue disorder caused by heterogeneous mutations in the genes encoding the transforming growth factor beta receptor one or two.[1] This syndrome is characterised by the triad of arterial tortuosity, aneurysm or dissections, hypertelorism, and bifid uvula or cleft palate.[2] Here, we present a patient prenatally diagnosed with complex congenital heart disease and confirmed with Loeys–Dietz syndrome after birth.

Case report

A 31-year-old pregnant woman was referred to our paediatric cardiology unit at the 36th week of gestation because of foetal congenital heart disease and dilatation of the pulmonary artery.

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The first foetal echocardiography revealed a huge aneurysm of the main pulmonary artery and complex congenital heart disease – double-outlet right ventricle and interruption of the aortic arch (Fig 1). Detailed multi-planar scanning showed that there was no pulmonary valve stenosis, because of no acceleration in pulmonic flow, and no absent pulmonary valve. Therefore, we suspected a connective tissue disorder, such as Marfan syndrome. The foetus was followed up weekly for foetal decompensation and signs of hydrops until the 39th week of gestation, and an elective caesarean section was then performed. The male infant weighed 2834 grams at birth. After delivery, the infant developed dyspnoea and was intubated for artificial ventilation. Subsequently, a cleft of the soft palate and bifid uvula were noted. To treat the interruption of the aortic arch, we started him on a prostaglandin infusion to maintain patent ductus arteriosus and on nitrogen inhalation to prevent pulmonary blood flow increase. Computed tomography and angiocardiography confirmed the heart

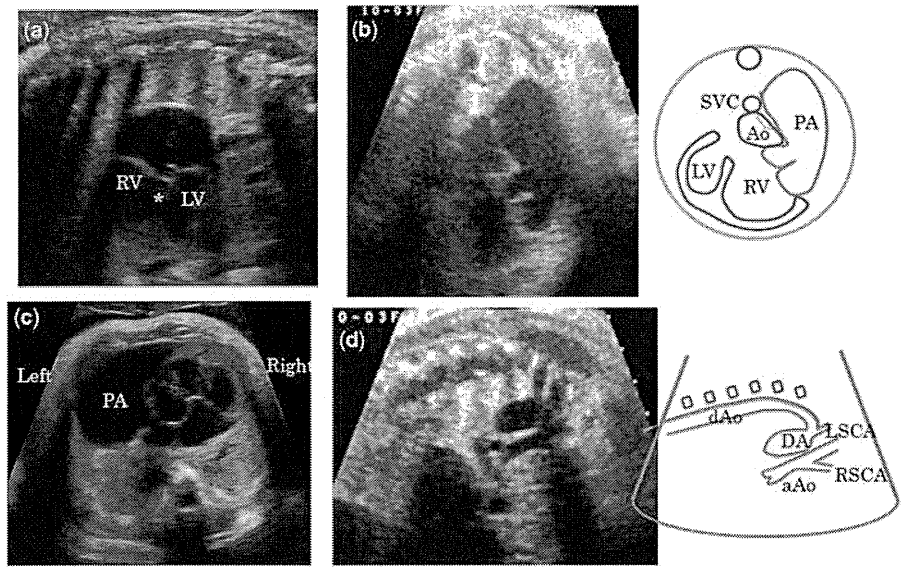

Figure 1.
Foetal echocardiography shows a large ventricular septal defect (*) of the double-outlet right ventricle (a), aneurysmal pulmonary artery (b, c), and interruption of the aortic arch (d). aAo = ascending aorta; Ao = aorta; DA = ductus arteriosus; dAo = descending aorta; LV = left ventricle; LSCA = left subclavian artery; PA = pulmonary artery; RSCA = right subclavian artery; RV = right ventricle; SVC = supra caval vein.

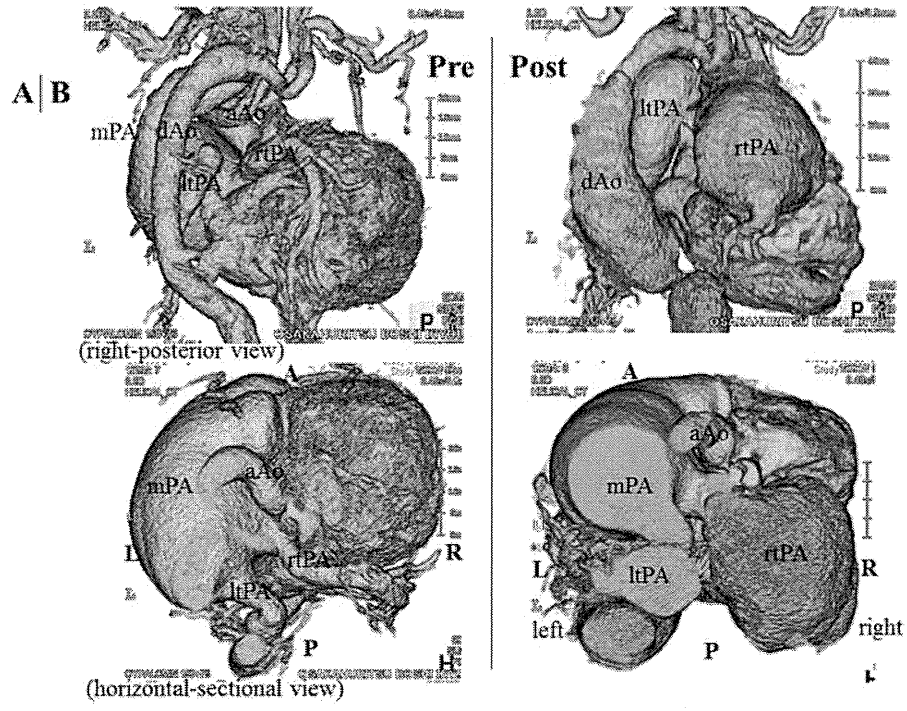

Figure 2.
Computed tomography (day 0) shows the interruption of the aortic arch and aneurysmal main pulmonary artery before operation (a). Computed tomography (day 18) shows progress of the significant expansion of the right and left pulmonary arteries and descending aorta after operation (b). A = anterior; aAo = ascending aorta; dAo = descending aorta; L = left; ltpa = left pulmonary artery; P = posterior; mPA = main pulmonary artery; rtPA = right pulmonary artery; R = right.

disease diagnosed prenatally (Fig 2a). Loeys–Dietz syndrome was strongly suspected because of the presence of cardiovascular lesions, thin skin, and facial appearance. On the 9th day, as the patient had suffered a pulmonary haemorrhage due to pulmonary blood flow increase, emergency bilateral

pulmonary artery banding was performed. However, during surgery, it became apparent that application of normal pulmonary artery banding was impossible because of the very thin condition of the pulmonary artery wall. Therefore, the surgeon performed bilateral banding with the clip, not the usual tape, but the banding was insufficient. This may be a reason why his haemodynamics and respiratory status were not subsequently stable. We again performed computed tomography, which showed a further significant expansion of the right pulmonary artery and descending aorta caused by the pressure of the expanded artery (Fig 2b). Therefore, we started internal use of Losartan. On the 21st day, he developed sudden hypotension and massive bleeding from the thoracic cavity, thought to be caused by right pulmonary rupture, and he died the same day. Subsequently, as the genetic analysis showed p.Thr200Pro (c.598A > C) mutation of the transforming growth factor beta receptor one, he was definitively diagnosed with Loeys–Dietz syndrome. The mutation was *de novo*.

Discussion

Loeys–Dietz syndrome is a recently described connective tissue disorder characterised by aggressive ascending aortic aneurysm and dissection. The clinical features are similar to Marfan syndrome,[3] but this is a more severe syndrome because life-threatening aortic dissection may occur even in early childhood.[4,5] Most patients have the triad of vascular aneurysms, hypertelorism, and bifid or broad uvula/cleft palate associated with variable features. Heterogeneous mutations in the genes encoding for transforming growth factor beta receptors one and two are a consistent finding among affected patients.

In addition, this syndrome shows various cardiovascular manifestations involving not only aortic lesions – such as distortion, aneurysm, and dissections – but also congenital heart diseases.[6] The case described in this report was also complicated with congenital heart disease. The patient's pulmonary artery showed an abnormal expansion because of his heart defect. That is, because he had an interruption of the aortic arch, much more blood than normal flowed through the pulmonary artery and the artery was stressed by "volume overload". Furthermore, the pulmonary artery was stressed by high "pressure overload" because the patient had double-outlet right ventricle and a large ventricular septal defect. It is thought that a pulmonary artery spread for both reasons from the foetal period.

Muramatsu et al[6] reported a case that was complicated with a ventricular septal defect and showed aortic and pulmonary expansion. It is thought that, in the Muramatsu case, the mechanism producing pulmonary artery dilatation was similar to that in the case reported herein. After birth, the patient's pulmonary blood flow increased due to the ventricular septal defect, which led to acute heart failure. He then underwent pulmonary artery banding on the 12th day. After surgery, however, the root of the main pulmonary artery, which was stressed by pressure, had spread in the shape of an aneurysm and intracardiac surgical repair, that is, closure of ventricular septal defect, was performed on the 42nd day. After the operation, the vascular expansion stopped worsening, and in conclusion they recommended early radical operation. However, because our case was a Fontan candidate, he required gradual surgery and radical operation was impossible in early infancy. Therefore, we performed bilateral pulmonary artery banding as a life-saving procedure, but, owing to mural abnormal thinning, the banding was insufficient, and his vascular expansion and thinning progressed, which finally led to explosion and bleeding to death.

In the case reported herein, significant pulmonary expansion from the foetal period led us to suspect a connective tissue disorder such as Marfan syndrome. Viassolo et al[7] reported a similar case in a female patient with Loeys–Dietz syndrome, who showed dilated aortic root from the foetal period. Only aortic dilatation was noted in screening foetal echocardiography at 19 gestational weeks and a connective tissue disease was suspected. She underwent genetic analysis and Loeys–Dietz syndrome was confirmed after birth. At present, the Viassolo case and the one we report herein are the only two cases showing a manifestation of Loeys–Dietz syndrome from the foetal period.

Some cases of Loeys–Dietz syndrome are complicated with congenital heart diseases.[2,6,8] However, those reported hitherto are associated with "simple" congenital heart diseases such as ventricular septal defect, atrial septal defect, patent ductus arteriosus, and aortic bicuspid valve. There is no previous report of Loeys–Dietz syndrome combined with complex congenital heart disease, such as double-outlet right ventricle and interruption of the aortic arch. In such a case, the cardiovascular lesion as an expansion of the great vessels, that is, the aorta or pulmonary artery, may be aggravated during the foetal period. Consequently, the foetus may die in utero. Even if they can be born, their great vessels are continuously or more strongly stressed after birth. Therefore, their arteries expand and finally explode, leading to an early death without undergoing any surgery.

This may be the reason why this is the first reported case of complex heart disease with Loeys–Dietz syndrome.