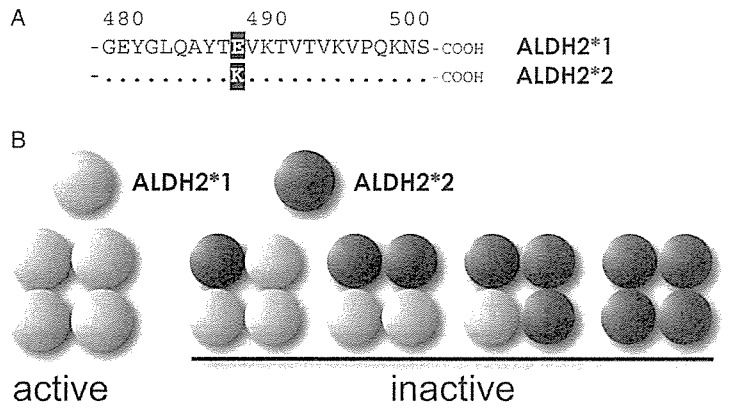

図4　ALDH2遺伝子多型と酵素活性
A：ALDH2のC末端領域のアミノ酸配列。ALDH2*1が活性型，ALDH2*2が不活性型。
B：4量体を形成して酵素活性を示す。不活性型のサブユニットが1つでもあると不活性型になるので，dominant-negativeに不活化する。ALDH2*1とALDH2*2が1：1の場合は酵素活性は16分の1になる。

ハイマー病の特異性を説明することはできない。最近，アミロイドβペプチド（Aβ）が，COX活性を阻害することが相次いで報告されている（Caseley et al, 2002；Strazielle et al, 2003；Crouch et al, 2005）。COXはミトコンドリアの内膜に存在する。最近になって，Aβはミトコンドリアにも存在することが明らかにされた。しかも，Aβをアミロイド前駆体蛋白（APP：amyloid precursor protein）から切り出すγ-secretaseがミトコンドリアにも存在することが報告され，ミトコンドリア内でAβが生成する可能性もありうる（Teng & Tang, 2005）。このAβによるCOX活性の阻害は，エネルギー代謝を低下させるだけでなく，電子伝達系を停止させることによって，活性酸素を放出させる可能性も同時に存在する。

II．酸化ストレスとアルツハイマー病

ミトコンドリア内では電子伝達系から漏れ出た電子が酸素と反応して，スーパーオキシドが生成する。このスーパーオキシドはミトコンドリア内のMnSOD（Mn-superoxide dismutase）によって速やかに過酸化水素に変換され，次いでカタラーゼあるいはグルタチオンペルオキシターゼによって水に変換される。ミトコンドリア内で生じたスーパーオキシドはそれほど酸化活性が強いわけではなく，直接DNAや蛋白質に損傷を与えるほどではない。しかし，このスーパーオキシドを解毒するMn-SOD酵素を欠損させると，神経系を中心に重大な影響があることから，スーパーオキシドが重大な神経細胞傷害性を与えていることはまちがいない（Melov et al, 1998）。しかし，このROSがどのようにして細胞を死に至らしめるかは不明であった。筆者らは，ALDH2の研究から，ROSとアルツハイマー病との関係を解明する糸口を見出した。

1．ALDH2遺伝子多型はアルツハイマー病の遺伝的危険因子

ミトコンドリアマトリクスには少なくとも2種類のアルデヒド脱水素酵素があり，その1つのアルデヒド脱水素酵素2（ALDH2）は，アルコール代謝の中心を担っている。エタノールは通常，アルコール脱水素酵素によってアセトアルデヒドに酸化される。ALDH2は低濃度のアセトアルデヒドを酢酸に変え，酢酸はアセチルCoAとなってエネルギー代謝経路へ導入される。ALDH2には北アジア人に特有の単一遺伝子多型（SNP）があり，エクソン12のSNPにより487番目のグルタミン酸がリジンへと変換し，不活型となる（図4A）。酵素活性型（487E）をALDH2*1と呼び，不活型（487K）をALDH2*2と呼ぶ。ALDH2では4量体が酵素活性を示し，ALDH2*2遺伝子産物はドミナントネガティブに酵素活性を低下させる（図4B）。したがって，ALDH2*1遺伝子産物とALDH2*2遺伝子産物が1：1でも酵素活性は16分の1になる。

筆者らは，ALDH2とアルツハイマー病の関連に注目し，大規模患者対照関連解析を行った（Kamino et al, 2000）。日本国内でも地域によって，ALDH2遺伝子のSNPの頻度が少しずつ異なる。そのため，対照の非アルツハイマー病健常人とアルツハイマー病患者の地域，性別，年齢を厳密に一致させた。すると，アルツハイマー病患者にはALDH2*2のSNP頻度が，男女差なく有意に高かった（Odd比1.6，p＝0.001）。さらに，ALDH2*2の頻度が偶然ではないことを確認するために，APOE-ε4の遺伝子と組み合わせて比較すると，APOE-ε4とALDH2の相乗効果がみられた（図5）。偽陽性なら相乗効果はみられないはずであり，ALDH2*2が危険因子であることがより確実になった。さらに，APOE-ε4とALDH2*2の相乗効果によって，発症年齢も有意に速められることが示された。

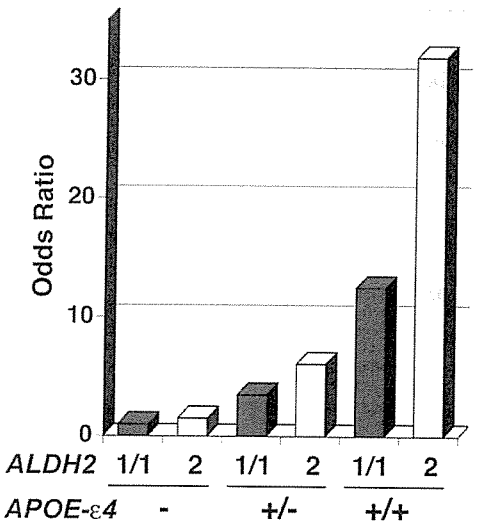

図5　孤発性アルツハイマー病における ALDH2 遺伝子多型と APOE-ε4 の相乗効果
アルツハイマー病447人と，地域，年齢，性別を一致させた対照447人のアルツハイマー病発症頻度を比較した。ALDH2の1/1はALDH2*1のホモ，2はALDH2*2を持つ人(ホモとヘテロの合計)。APOE-ε4(−)はなし，(+/−)はヘテロ，(+/+)はホモ。ALDH2*2を持ち，APOE-ε4ホモの人はオッズ比にして最大31の発症頻度となる。対応する人は日本人の1%程度。

これらの結果は，病理診断によって確定した患者試料を用いて，筑波大の玉岡らによってALDH2＊2がアルツハイマー病の危険因子であること，APOE-ε4との相乗効果があることの再現性が確認された(Tamaoka et al, 2003, 日本痴呆学会)。

2. 4-hydroxy-2-nonenal の役割—ALDH2*2 がアルツハイマー病の危険因子となる分子機構

次に，ALDH2*2がアルツハイマー病の発症に関わる原因を突き止めるために，まず，ALDH2*2を持つ人の表現型を明らかにしようとした。一般的な調査では，遺伝子によって起こされる遺伝的要因による表現型なのか，遺伝子に影響される生活習慣によって起こされる表現型なのかを区別することは難しい。ALDH2の遺伝子多型では，遺伝子型と飲酒の関連は非常に相関が強くて，ALDH2*1ホモの人は飲酒量が多い。すなわち，飲酒という生活習慣の違いによって生じた表現型と，直接的な遺伝子により生じた表現型の区別が通常はできない。

長寿医療研究センターの疫学研究部(下方浩史部長)の大規模疫学調査では，飲酒量も含めた生活習慣についての調査も同時に行っている(Shimokata et al, 2000)。地域住民の住民台帳から無作為抽出して対象者を選定しているので，厳密に偏りのない中立的な集団が対象である。2,259人を対象にALDH2遺伝子多型を調べ，飲酒量の効果を補正し，ALDH2*2の表現型の特徴を探った。すると，ALDH2*2を持つ女性群で飲酒量とは無関係に，血中過酸化脂質濃度が有意に高いことが判明した(Ohsawa et al, 2003)。男性の場合，飲酒量とALDH2遺伝子多型の相関があまりに強く，飲酒量を補正しきれなかったので有意差が出なかったのかもしれない。

アルツハイマー病患者脳においても，過酸化脂質の蓄積が報告されている(Montine et al, 2002)。そこで，飲酒の結果ではなくALDH2*2の遺伝子の直接効果によって，酸化ストレスが亢進していることを推定した。ミトコンドリアから発生したスーパーオキシドが，不飽和脂肪酸と反応して過酸化脂質が生成する。過酸化脂質からは自然反応でtrans-4-hydroxy-2-nonenal(4-HNE)が生成される。4-HNEはアルデヒド基を持ち，反応性が高く，蛋白質を修飾して失活させるので，低濃度でも細胞毒性は高く，細胞を死に導く。しかも，アルツハイマー病では4-HNEが蓄積していることが報告されている。そこで，以下の作業仮説を立てた(図6)。(1) ALDH2は4-HNEのアルデヒド基を酸化して解毒する役割がある，(2) ALDH2の酵素活性が低下している人では4-HNEを十分酸化することができないので，4-HNEが相対的に蓄積する，(3) 4-HNEの蓄積によりミトコンドリア呼吸鎖酵素活性が阻害され，ROS出現の頻度が高くなる，(4) ROSにより過酸化脂質が生成し，自然反応で4-HNEが生成される。

以上の作業仮説を証明するために，マウス型(ラット型も同じ)ALDH2*2遺伝子を作製し，ラットPC12細胞に導入した。ALDH2*2遺伝子を導入して発現させた細胞では，ALDH2活性が抑制された。そして，ALDH2酵素活性低下細胞では，4-HNEによって容易に細胞が死滅した(図7)。また，アンチマイシンAによってミトコンドリアから活性酸素を放出させると，ALDH2活性欠損株では4-HNEが蓄積した(図8)。以上の結果から，ALDH2は細胞内で4-HNEを酸化し，解毒することが証明された(Ohsawa et al, 2003)。アルツハイマー病患者脳で4-HNEが蓄積する原因はALDH2酵素欠損だけとは限らず，何らかの他の原因によって酸化ストレスの亢進した結果，4-HNEが蓄積すると考えてもよい。現在，ALDH2*2を導入したトランスジェニックマウスを作製し，神経細胞を含めた老化現象が促進されていることを確認している。

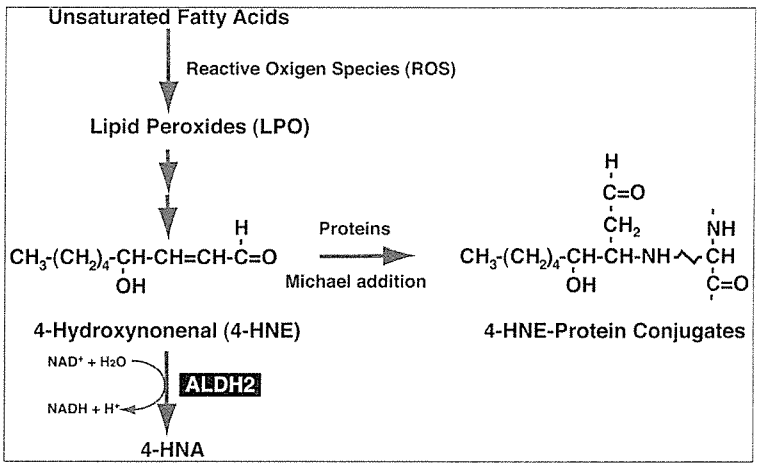

図6 trans-4-hydroxy-2-nonenal (4-HNE) の形成機構と ALDH2 による酸化
4-HNE は過酸化脂質より生成し，ALDH2 によって酸化される。4-HNE は蛋白質や核酸を修飾し，不活化させるので細胞傷害性が高い。

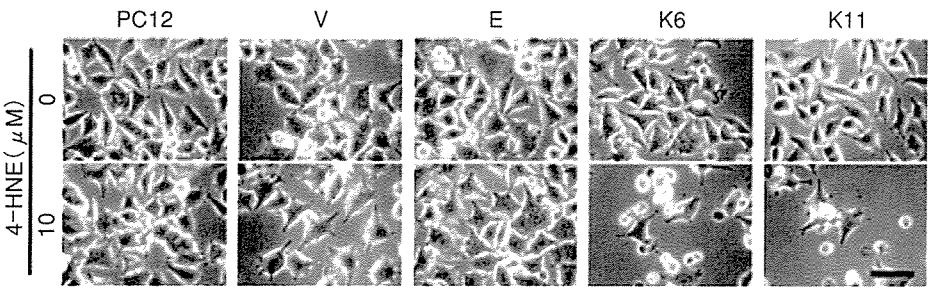

図7 マウス型 ALDH2*2 を導入したラット PC12 の 4-HNE に対する脆弱性
PC12 にマウス型 aldh2*2 遺伝子を導入し，ALDH2 活性を低下させた（クローン K6，K11）。4-HNE を加えると ALDH2 活性を持つクローンではほとんど死なないのに対し，ALDH2 酵素活性のない K6 と K11 は容易に死滅した。

韓国でも ALDH2 遺伝子多型とアルツハイマー病の関連に興味がもたれ，認知能力と ALDH 活性の相関関連はないという結果が報告されている（Kim et al, 2004）。アルツハイマー病 60 人を解析し，統計的有意差が認められなかったとしているが，人数の点から有意差が出ないのは当然であろう。

従来から ALDH2 はアルコール代謝との関連でのみ議論されてきた。しかし，飲酒をしない他の動物でも同じ遺伝子が存在するので，ALDH2 には飲酒とは無関係の本来の機能があるはずである。筆者らの研究結果からは，ALDH2 は酸化ストレスの防御機構のひとつと考えるのが妥当である（Ohta et al, 2004）。

3. ABAD と Aβ

最近になって，ミトコンドリア内のアルコール脱水素酵素がアルツハイマー病に重要な役割を果たしていることが明らかとなった。アルツハイマー病において，ミトコンドリアの機能低下はアミロイドβ蛋白（Aβ）による神経細胞毒性での顕著な特徴として認められていたが，その分子機序については不明であった。アミロイドβ蛋白結合アルコール脱水素酵素（ABAD）が，Aβとミトコンドリア毒性を直接的に結びつける分子であることが示された。AD 患者およびトランスジェニックマウスのミトコンドリアにおいて，Aβが ABAD と結合することが明らかにされた（Lustbader et al, 2004）。NAD^+（ニコチンアミドアデニンジヌクレオチド）存在下で行った ABAD-Aβ複合体の結晶構造解析によると，Aβと結合した ABAD では結合部位の立体構造が大きく変化しており，NAD^+ と結合できなくなっていた。ABAD-Aβ相互作用を特異的に阻害する ABAD 由来ペプチドが，神経細胞において Aβにより誘導されるアポトーシスとフリーラジカルの生成を抑制することが示された。Aβ産生を高めると考えられる変異型アミロイド前駆蛋白と ABAD を，同時に過剰発現させたトランスジェニックマウスにおいて，神経細胞における酸化ストレスの増加と記憶障害の発現が見出された。これらの結果より，ミトコンドリアに

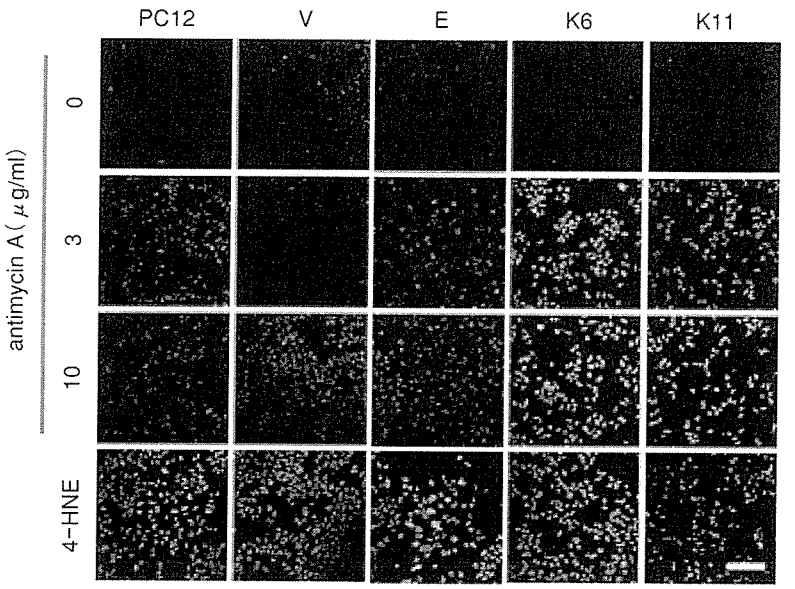

図8 ALDH2活性と4-HNEの蓄積
図7と同じ細胞でアンチマイシンAにより呼吸鎖から活性酸素を放出させた。下段の4-HNEは外から加えたコントロール。4-HNEを特異的抗体で検出した。ミトコンドリアから放出された活性酸素により4-HNEが生成し，ALDH2活性が消失したクローンK6，K11では，4-HNEが蓄積している。

おけるABADとAβとの結合とそれに引き続くミトコンドリア機能の破綻が，ADでの神経細胞死発現に密接に関わることが示されるとともに，ABAD-Aβ相互作用がADの治療標的となりうることが示唆された。

さらに，APPに変異があり高発現しているトランスジェニックマウスにABADを発現させるとROSが発現し，ATPが減少し，アポトーシスが誘導された(Takuma et al, 2005)。しかもROSの出現はCOXの活性低下と相関していた。COXの阻害剤で活性を低下させるとROSの出現はさらに顕著になるので，COXの低下とABADとAβの結合がROSの出現を促進しているようである。

以前からAβの毒性はROSにより増強されることが報告されている(Behl et al, 1994)。最近の報告は以前の報告と矛盾せず，分子機構がより詳細に明らかにされつつある。

Ⅲ．ミトコンドリアDNAの体細胞変異とアルツハイマー病

ミトコンドリアDNA(mtDNA)は加齢に伴って，酸化ストレスにより体細胞変異が蓄積する。神経細胞は，酸素消費に伴いROSが発生しやすい組織であり，mtDNAに変異が蓄積しやすい細胞である。同時に，神経細胞自身は増殖しないのに対して，継続的にmtDNAは複製と消失を続ける。そのため，神経細胞のmtDNAの体細胞変異はより蓄積されやすい。通常，体細胞変異では変異mtDNAと正常mtDNAが混在することになるが，特定の変異だけが蓄積する場合でないと検出が難しい。

アルツハイマー病患者脳由来のmtDNAを持ち，癌細胞の核を持つ人口細胞を用いた実験では，Aβの毒性が強くなっており，mtDNAの変異蓄積が検出できない程度でも，変異mtDNAがAβの細胞毒性を助長しているようである(Cardoso et al, 2004)。

さらに，アルツハイマー病患者脳のmtDNAを調べると，検出できるくらいに変異が蓄積している場所があり，しかもそこは転写制御領域であった。アルツハイマー病患者脳でみられた変異の蓄積によって転写が低下し，COXの活性が低下していた(Coskun et al, 2004)。mtDNAの体細胞変異によって，COXなどのミトコンドリア機能が低下するのも，アルツハイマー病の発症の一原因と言ってもいいだろう。

おわりに

ミトコンドリアはエネルギー代謝，アポトーシス，カルシウム調節，活性酸素の発生源であり，細胞死と様々な点で関連している。しかし，アルツハイマー病における神経細胞死とどのように関与するかという分子機構は，未解決の問題であった。アミロイドβがミトコンドリア内でCOX活性を阻害したり，アルコール脱水素酵素と結合してROSを発生させるという報告は，アルツハイマー病の発症にミトコンドリアが直接的な役割を果たしている証拠であろう。また，筆者らのCOXの分子集合に関与するMIRTDが，アルツハイマー病患者脳には少ないという結果も一致した結

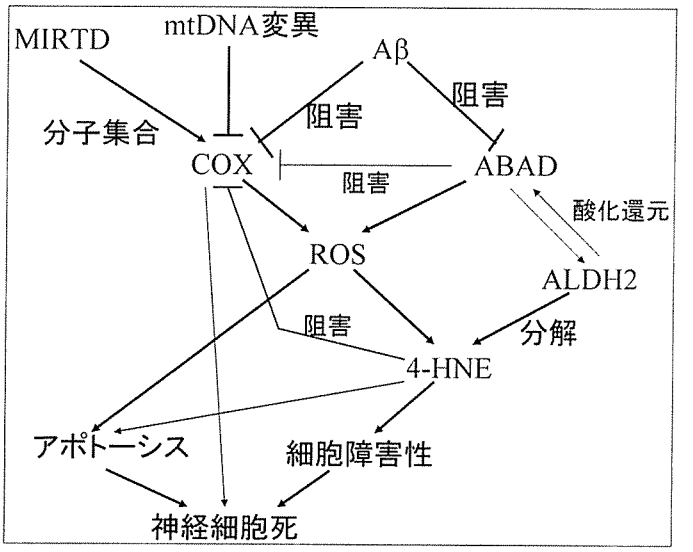

図9 ミトコンドリア内における MIRTD, COX, Aβ, ABAD, 4-HNE, ALDH2 の相互関係

MIRTD が COX の分子集合を司るので MIRTD 減少細胞では COX 活性が低下する。Aβ は COX の活性を阻害し，ROS を発生させる。mtDNA 変異は COX 活性を低下させ ROS を促進する。一方，Aβ は ABAD（Aβ結合アルコール脱水素酵素）と結合して ABAD の活性を低下させ，ROS を発生させる。ROS は過酸化脂質を経由して毒性の強い 4-HNE を生成する。ALDH2 は 4-HNE を解毒する。

果，ALDH2 酵素活性欠損がアルツハイマー病の危険因子となるのは，ALDH2 がミトコンドリア内で酸化ストレスの防御機構として働いているからである。図9にミトコンドリア内における MIRTD, COX, Aβ, ABAD, 4-HNE, ALDH2 の相互関係をまとめた。アルツハイマー病の原因は，最終的にミトコンドリアのエネルギー代謝を低下させ，ROS を生じさせることが本質的な原因かもしれない。今後の研究成果に期待したい。

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Abstract

Contribution of dysfunction of mitochondria and oxidative stress in the pathogenesis of Alzheimer's disease

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Mitochondrion is a multifunctional organelle. Apoptosis as well as necrosis is closely related with mitochondrial functions. Damage to mitochondria causes a decline in ATP synthesis and an increase in the generation of reactive oxygen species (ROS). ROS damage various molecules including DNA, protein and lipid and induce apoptosis. Evidence has recently emerged that oxidative stress is involved in the pathogenesis of Alzheimer's disease (AD). Moreover, it has been shown that amyloid β peptide is involved in enhancing oxidative stress.

Here, the author focus on the involvement of dysfunction in mitochondria with AD and review recent approaches to the molecular pathogenesis of AD.

First, the author would like to describe the involvement of deficiency of molecular assembly of cytochrome c oxidase (COX). Dihydrolipoamide succinyltransferase (DLST) is a subunit-enzyme of the α-ketoglutarate dehydrogenase complex of the Krebs cycle. While studying how the *DLST* genotype contributes to the pathogenesis of AD, we found a novel mRNA that is transcribed starting from intron 7 in the *DLST* gene. The novel mRNA level in the brain of AD patients was significantly lower than that of controls. The truncated gene product (designated MIRTD) localized to the intermembrane space of mitochondria. To investigate the function of MIRTD, we established human neuroblastoma SH-SY5Y cells expressing a maxizyme, a kind of ribozyme, that specifically digests the MIRTD mRNA. The expression of the maxizyme specifically eliminated the MIRTD protein and the resultant MIRTD-deficient cells exhibited a marked decrease in the amounts of subunits of the COX complex of the mitochondrial respiratory chain, resulting in a decline of activity. A pulse-label experiment revealed that the loss of the subunits is a post-translational event. Thus, the *DLST* gene is bifunctional and MIRTD transcribed from the gene contributes to molecular assembly of the mitochondrial respiratory complex, including COX.

Second, the author review on oxidative stress caused by a deficiency of mitochondrial algehyde dehydrogenase 2 (ALDH2). ALDH2 is involved in ethanol metabolism by playing a major role in acetaldehyde detoxification. A polymorphism of the ALDH2 gene is specific to north Asians. Sensitivity to ethanol is highly associated with this polymorphism (*ALDH2*2* allele), which is responsible for a deficiency of ALDH2 activity. We at first show that this deficiency influences the risk for late-onset Alzheimer's disease (LOAD) by a case-control study in a Japanese population. In a comparison of 447 patients with sex, age and region-matched non-demented controls, the genotype frequency for carrying the *ALDH2*2* allele was significantly higher in the patients than in the controls ($p=0.001$). Next, we examined the combined effect of the *ALDH2*2* and apolipoprotein E 4 allele (*APOE-* ε *4*), which has been confirmed to be a risk factor for LOAD. The *ALDH2*2* allele more significantly affected frequency and onset-age in patients with *APOE-* ε *4* than without. These results indicate that the ALDH2 deficiency is a risk factor for LOAD, acting synergistically with the *APOE-* ε allele. Next, to elucidate the molecular mechanism involved, we obtained ALDH2-deficient cell lines by introducing mouse mutant *Aldh2* cDNA into PC12 cells. We speculate that ALDH2 may function to oxidize toxic aldehyde derivatives. Then, we found that the ALDH2-deficient transfectants were highly vulnerable to exogenous 4-hydroxy-2-nonenal, an aldehyde derivative generated from peroxidized fatty acids. In addition, the ALDH2-deficient transfectants were sensitive to oxidative insult induced by antimycin A, accompanied by an accumulation of proteins modified with 4-hydroxy-2-nonenal. Mitochondrial ALDH2 functions as a protector against oxidative stress.

Finally, the author would like to review on the direct involvement of Aβ with mitochondrial dysfunctions, through inhibition of COX activity and enhancement of oxidative stress via Aβ-binding alcohol dehydrogenase (ABAD) which is located in mitochondria.

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ORIGINAL ARTICLE

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Association of alcohol dehydrogenase 2*1 allele with liver damage and insulin concentration in the Japanese

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Abstract The Japanese have a polymorphism in the alcohol dehydrogenase 2 gene (*ADH2*). The alleles of *ADH2* (*ADH2*1* and *ADH2*2*) encode more active and less active forms for ethanol metabolism, respectively. We examined whether liver damage and the insulin–glucose axis vary according to *ADH2* genotype in the Japanese. The 2,232 subjects (1,126 men and 1,106 women) were recruited from a population-based prospective cohort study. Clinical evaluations including alcohol consumption, percentage of alcohol drinkers, plasma glucose, HbA1c, insulin, AST, ALT, γ-GTP, and prevalence of diabetes were compared among the *ADH2* genotypes. The percentage of drinkers, alcohol consumption, AST, ALT, and γ-GTP were higher in group *ADH2*1/1* than in group *ADH2*1/2* or *ADH2*2/2* (all $P<0.05$). Hence, *ADH2*1/1* is associated with excess alcohol intake and liver disorders. However, the prevalence of diabetes did not differ among the three groups. For the glucose–insulin axis, we examined subjects who did not receive insulin therapy or oral anti-diabetes medication. While amounts of alcohol consumed and glucose levels were nearly the same between *ADH*1/2* and *ADH2*2/2*, insulin concentrations were lower in *ADH2*2/1* than in *ADH2*2/2* ($P<0.05$ in men). This finding suggests that the *ADH2*1* allele is associated with a lower insulin concentration when alcohol intake is light or moderate. It also suggests that the genetic effect of *ADH2*1* plays an important role in alcohol drinking behavior and in the occurrence of liver injury, but the effect is so mild that it does not influence the glucose–insulin axis or prevalence of diabetes.

Keywords Alcohol dehydrogenase 2 · ADH2 · Diabetes · Insulin resistance · Liver dysfunction · Alcohol · Prospective cohort study

Abbreviations: ALDH: Aldehyde dehydrogenase · ADH: Alcohol dehydrogenase · PCR: Polymerase chain reaction

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Introduction

A reduced incidence of type 2 diabetes has been observed among drinkers in several large prospective studies. Conigrave et al (2001) reported a 12-year prospective study in a cohort of 46,892 US male health professionals, in which 1,571 new cases of type 2 diabetes were reported. The frequency of alcohol consumption was inversely associated with diabetes. Hu et al (2001) reported a large cohort study of 84,941 female nurses from 1980 to 1996, in which abstinence from alcohol use was associated with a significantly increased risk of diabetes. In contrast, other studies (Holbrook et al 1990) have shown an increased risk of diabetes among a proportion of subjects in the top alcohol consumption category. In Japanese men, Tsumura et al (1999) reported that heavy drinking is associated with an increased risk of type 2 diabetes, while moderate drinking is associated with a decreased risk of type 2 diabetes, showing a U-shaped relationship.

The genotypes involved in ethanol metabolism are now known to be associated not only with drinking, but also with longevity and oxidative stress parameters (Ohsawa et al 2003). In Japanese, the pharmacokinetics of alcohol metabolism have been well studied. Alcohol dehydrogenase (ADH) is one of the key enzymes in alcohol metabolism. Class I ADH isoenzymes, encoded

by *ADH1*, *ADH2* and *ADH3*, form dimers among the isoenzymes and oxidize ethanol and other small aliphatic alcohols (Borson et al 1988). About 85% of the Japanese population are carriers of the β2-subunit encoded by the *ADH2*2* allele, while isoenzymes with the β2-subunit have been found in only 5% or less of Europeans and white Americans. The β1- and β2-subunits differ by only one amino acid residue: Arg-47 in the NAD(H) pyrophosphate-binding site is substituted with His-47 in the β2-subunit. ADH2 functions as a dimer and the $\beta2\beta2$ dimer exhibits about 100 times more catalytic activity for ethanol oxidation than the $\beta1\beta1$ dimer at physiological pH (Borson et al 1988), whereas the $\beta1\beta2$ heterodimer exhibits nearly the same activity as the $\beta1\beta1$ homodimer. Thus, relative enzymatic activities of *ADH2*1/1*:*ADH2*1/2*:*ADH2*2/2* can be estimated as 1:26:100 if a dimer were to form between the subunits of ADH2*1 and ADH2*2 (Borson et al 1988; Yoshida et al 1981).

Several studies (Higuchi et al 1996; Yamauchi et al 2001) have reported that the *ADH2* genotype is associated with excess alcohol intake and alcohol-related disorders in the Japanese population. We have previously reported that the *ADH2* genotype affected LDL-cholesterol levels and the occurrence of cerebral infarction in a community-dwelling Japanese population (Suzuki et al 2004). We therefore examined whether the glucose–insulin axis or prevalence of diabetes is associated with the *ADH2* genotype in the same Japanese population.

Research design and methods

The National Institute for Longevity Sciences–Longitudinal Study of Aging (NILS–LSA), a population-based prospective cohort study of aging and age-related diseases, was begun in 1997 (Ohsawa et al 2003; Shimokata et al 2000; Yamada et al 2002). All participants were independent residents of the Aichi prefecture in Japan. Residents aged 40–79 years old were randomly selected from the register in co-operation with the local government.

The area of study is located in the south of Nagoya City. It is a commuter town and contains an industrial area belonging to the Toyota group, but it has many orchards and farms, so it has both urban and rural characteristics. This area is geographically located in the center of Japan, and its climate is average for Japan. We examined a representative sample of the area's population via a national postal questionnaire of prefecture-stratified random samples of 3,000 households from all prefectures in Japan, and previously showed that the lifestyle of people in this area was the most typical of all areas in Japan.

The sample consisted of 2,232 subjects (1,126 men and 1,106 women) who were randomly recruited. We refer to them as "subjects-1." Subjects-1 was stratified by both age and sex. Randomly selected men and women were invited, by mail, to attend an explanatory meeting. At the meeting, the procedures for each examination and follow-up schedule were fully explained. Written informed consent to the entire procedure was obtained from each participant. Participants in the present study were recruited from subjects examined in 1997–1999. The study protocol was approved by the Committee on the Ethics of Human Research of National Chubu Hospital and the National Institute for Longevity Sciences.

Descriptions of the physical examinations performed have been published before (Ohsawa et al 2003; Shimokata et al 2000; Yamada et al 2002). In brief, lifestyle, medical history and prescribed drugs were examined by questionnaire. Anthropometric measurements were taken by a physician. A drinker is defined as a subject who has drunk more than 5 g of alcohol on average per day during the past year. Amounts of alcohol consumed were carefully examined by taking pictures before and after drinking as well as with questionnaires. The percentage of non-smokers to smokers was also noted.

Venous blood was collected early in the morning after at least 12 h fasting. The mean of two determinations of blood chemistry data was obtained for each participant. Clinical evaluations included gender, age, height, body-mass index, smoker status, alcohol consumption, percentage of alcohol drinkers, and blood chemistry (fasting plasma glucose (FPG), HbA1c, insulin, AST, ALT, and γ-GTP levels). Diagnosis of diabetes was based on medical records, or it was defined as a FPG concentration greater than 126 mg/dl or an HbA1c of more than 6.5%, and/or if medication was taken to lower the blood glucose level. Namely, not all subjects whose FPG level was greater than 110 mg/dl did not receive the 75 g oral glucose tolerance test according to the criteria of the Japan Diabetes Society. In the analysis of glucose–insulin associated parameters, to exclude the effect of medications, the diabetic patients who received insulin therapy or oral medications for diabetes were excluded from subjects-1, and the remaining subjects were defined as the "subjects-2" group.

Genotyping of *ADH2*

Samples of DNA were isolated from peripheral blood cells. Genotypes were determined with a fluorescence-based allele-specific DNA primer-probe assay system (Toyobo Gene Analysis, Tsuruga, Japan). To determine the genotype with the G214A substitution (Arg-47-His), the polymorphic region of *ADH2* was amplified by polymerase chain reaction (PCR) with an antisense primer labeled at the 5′ end with biotin (5′-GATGGTGGCTGTAGGAATCTG-3′) and a G allele-specific sense primer labeled with FITC (5′-CCACGTGGTCATCTGTNCG-3′) or A allele-specific sense primer labeled with Texas red (5′-AACCACGTGGTCATCTGTNTG-3′).

Table 1 Comparison of parameters among three groups of men (subjects-1), divided according to *ADH2* genotype. Right columns indicate *P*-values of statistical differences between two groups

Variables	Men			*P*-value		
ADH genotype	2/2	1/2	1/1	2/2 vs. 1/2	2/2 vs. 1/1	1/2 vs. 1/1
Subjects-1 $n=$	689	378	59			
Age (years)	59.5 ± 0.4	58.9 ± 0.6	58.0 ± 1.4	n.s.	n.s.	n.s.
Height (cm)	164.4 ± 0.2	164.7 ± 0.3	164.6 ± 0.8	n.s.	n.s.	n.s.
BMI	23.0 ± 0.1	22.8 ± 0.1	22.9 ± 0.4	n.s.	n.s.	n.s.
Smoking (%)	61/39	63/37	63/37	n.s.	n.s.	n.s.
Alcohol (g/day)	28.8 ± 1.4	29.5 ± 1.9	44.5 ± 4.8	n.s.	0.0049**	0.0102**
Drinkers (%)	67.0	67.1	85.5	($P < 0.0175$)		
AST (IU/l)	26.6 ± 0.7	26.6 ± 0.9	33.6 ± 2.3	n.s.	0.0038**	0.0049**
ALT (IU/l)	27.1 ± 0.9	26.8 ± 1.2	34.3 ± 3.0	n.s.	0.02*	0.02*
γ-GTP (IU/l)	58.2 ± 3.1	57.3 ± 4.1	80.3 ± 10.5	n.s.	0.04*	0.04*
Diabetics (%)	13.3	13.3	13.6	n.s.	n.s.	n.s.

AST 2/2 ± 1/2 vs. 1/1, $P < 0.0033$; ALT 2/2 ± 1/2 vs. 1/1, $P < 0.02$; γ-GTP 2/2 ± 1/2 vs. 1/1, $P < 0.04$; drinkers 2/2 ± 1/2 vs. 1/1, $P < 0.005$; alcohol 2/2 ± 1/2 vs. 1/1, $P < 0.005$

*$P < 0.05$

**$P < 0.01$

Statistical analysis

Data are presented as means ± SE. The statistical significance of any difference in mean values and frequencies was determined with the Student's *t*-test or the Tukey–Kramer test. We used a one-way analysis of variance to test for overall differences among multiple groups, and the Fisher LSD post hoc test to identify which group differences accounted for the significant *P*-value. The significance of deviation from Hardy–Weinberg equilibrium was analyzed using the chi-square test. A *P*-value of < 0.05 was considered statistically significant.

Results

Influence of *ADH2* genotypes on drinking behavior and liver function

Among the 2,232 subjects, 1,355 (men 689, women 666) had the *ADH2*2/2* genotype, 759 (men 378, women 381) had the *ADH2*2/1* genotype, and 118 (men 59, women 59) had the *ADH2*1/1* genotype. The *ADH2*2/2*, *ADH2*2/1*, and *ADH2*1/1* genotypes were in Hardy–Weinberg equilibrium. There was no gender difference.

First, we compared the percentage of drinkers dependent upon *ADH2* genotype. The percentage of drinkers was significantly higher in both men and women in the *ADH2*1/1* group, showing overall differences among the groups (Table 1 and Fig. 1a). The difference was statistically significant according to the Fisher LSD post hoc test in men ($P < 0.0175$), women ($P < 0.0166$), and total subjects-1 ($P < 0.0033$) (Table 1). Moreover, amounts of alcohol consumed were much higher in the *ADH2*1/1* group than the other *ADH2* groups in men and total subjects-1 ($P < 0.01$ in *ADH2*2/2* vs. *ADH2*1/1* and $P < 0.05$ in *ADH2*1/2* vs. *ADH2*1/1*) (Tables 1, 3 and Fig. 1b). On the other hand, no significant difference in alcohol consumption among *ADH2*1/1* and the other groups was found in women, probably because much less alcohol was consumed by women than men (Table 2 and Fig. 1b). For smoking (percentage of non-smokers to smokers), there was no difference according *ADH2* genotype in men and in women.

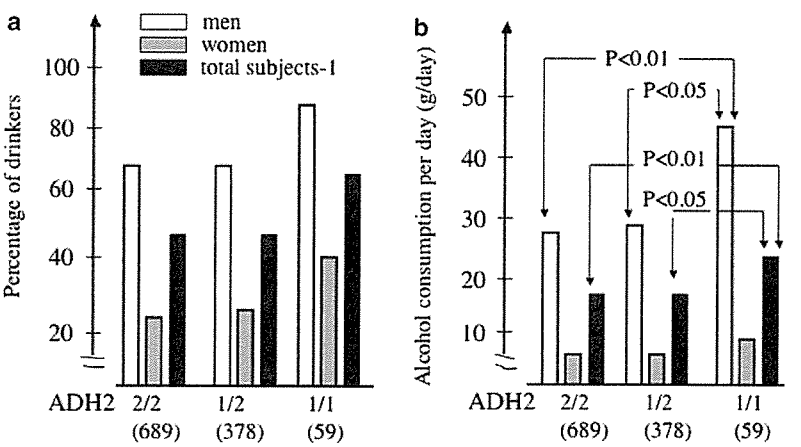

Fig. 1a, b Correlation of *ADH2* genotype with alcohol drinking behavior. **a** Percentage of drinkers in three groups based on *ADH2* genotype. Values in parentheses indicate the total number of subjects (*white bars* men, *gray bars* women, and *black bars* total subjects). **b** Average amounts of alcohol consumed per day. Subjects in the *ADH2*1/1* group drink more alcohol than those in the *ADH2*2/2* and *ADH2*1/2* groups

Table 2 Comparison of parameters among three groups of women (in subjects-1), divided according to the three *ADH2* genotypes. Right columns indicate *P*-value of statistical difference between each two group

Variables	Women			*P*-value		
ADH genotype	2/2	1/2	1/1	2/2 vs. 1/2	2/2 vs. 1/1	1/2 vs. 1/1
Subjects-1 $n=$	666	381	59			
Age (years)	59.4 ± 0.4	59.1 ± 0.6	60.0 ± 1.4	n.s.	n.s.	n.s.
Height (cm)	151.3 ± 0.2	151.1 ± 0.3	151.1 ± 0.8	n.s.	n.s.	n.s.
BMI	23.0 ± 0.1	22.7 ± 0.2	23.1 ± 0.4	n.s.	n.s.	n.s.
Smoking (%)	93/7	93/7	92/8	n.s.	n.s.	n.s.
Alcohol (g/day)	5.2 ± 0.6	5.4 ± 0.8	6.4 ± 2.0	n.s.	n.s.	n.s.
Drinkers (%)	22.9	25.5	39.7	< 0.0166		
AST (IU/l)	24.5 ± 0.6	23.5 ± 0.7	23.3 ± 1.8	n.s.	n.s.	n.s.
ALT (IU/l)	21.2 ± 0.8	20.1 ± 1.0	18.9 ± 2.5	n.s.	n.s.	n.s.
γ-GTP (IU/l)	27.9 ± 1.1	28.5 ± 1.4	29.4 ± 3.6	n.s.	n.s.	n.s.
Diabetics (%)	9.16	10.5	6.78	n.s.	n.s.	n.s.

Drinkers 2/2 ± 1/2 vs. 1/1, $P < 0.01$

Next, we compared blood parameters of liver function, namely AST, ALT, and γ-GTP activities. In men, levels were significantly higher in the *ADH2*1/1* group than the other two *ADH2* groups (Table 1, AST; $P < 0.01$ in *ADH2*2/2* vs. *ADH2*1/1* and $P < 0.01$ in *ADH2*1/2* vs. *ADH2*1/1*. ALT; $P < 0.05$ in *ADH2*2/2* vs. *ADH2*1/1* and $P < 0.05$ in *ADH2*1/2* vs. *ADH2*1/1*. γ-GTP; $P < 0.05$ in *ADH2*2/2* vs. *ADH2*1/1* and $P < 0.05$ in *ADH2*1/2* vs. *ADH2*1/1*), indicating that more alcohol intake in the *ADH2*1/1* group causes damage to the liver. On the other hand, no significant difference was found in women (Table 2); nevertheless the *ADH2*1/1* group consumed more alcohol than the other groups, probably because women drink less than men.

In subjects-1, the percentage of those with diabetes was compared among the three ADH2 genotypic groups. However, there was no statistical difference in the prevalence of diabetes among the three groups (men; *ADH2*2/2*:13.3%, *ADH2*1/2*:13.3%, and *ADH2*1/1*:13.6%, women; *ADH2*2/2*:9.2%, *ADH2*1/2*:10.5%, and *ADH2*1/1*:6.8%, total subjects-1; *ADH2*2/2*:11.2%, *ADH2*1/2*:11.9%, and *ADH2*1/1*:10.2%) (Tables 1, 2, 3).

Influence of *ADH2* genotype on fasting insulin concentration

We tried to clarify the correlation of insulin concentration with *ADH2* genotype. To exclude the effect of medication, subjects were limited to those (subjects-2) not treated with insulin therapy and/or with oral medications for diabetes. Although habits or behaviors generally depend upon genetic factors, we would like to distinguish the genetic effects from the secondary results of alcohol consumption. Since the frequency of drinking and the amountsof alcohol consumed were the same in the *ADH2*1/2* and *ADH2*2/2* groups (Fig. 1 and Tables 1, 2, 3), we compared fasting insulin concentrations between these two groups. Insulin levels were lower in the *ADH2*1/2* than *ADH2*2/2* group in total subjects-2 ($P < 0.02$). In men, insulin levels were lower in the *ADH2*1/2* than *ADH2*2/2* group ($P < 0.05$), while in

Table 3 Comparison of parameters among three groups of total subjects-1 divided according to *ADH2* genotype. Right columns indicate *P*-values of statistical differences between two groups

Variables	Total (men + women)			*P*-value		
ADH genotype	2/2	1/2	1/1	2/2 vs. 1/2	2/2 vs. 1/1	1/2 vs. 1/1
Subjects-1 $n=$	1,352	756	118			
Age (years)	59.4 ± 0.3	59.0 ± 0.4	59.0 ± 1.0	n.s.	n.s.	n.s.
Height (cm)	158.2 ± 0.2	158.1 ± 0.3	156.8 ± 0.8	n.s.	n.s.	n.s.
BMI	23.0 ± 0.1	22.7 ± 0.1	23.1 ± 0.3	n.s.	n.s.	n.s.
Smoking (%)	77/23	78/22	78/22	n.s.	n.s.	n.s.
Alcohol (g/day)	17.2 ± 0.9	17.6 ± 1.1	24.9 ± 2.8	n.s.	0.0089**	0.0158**
Drinkers (%)	45.4	45.6	62.0	< 0.0033		
AST (IU/l)	25.6 ± 0.4	25.0 ± 0.6	28.3 ± 1.4	n.s.	n.s.	0.0383**
ALT (IU/l)	24.2 ± 0.6	23.4 ± 0.8	26.5 ± 2.0	n.s.	n.s.	n.s.
γ-GTP (IU/l)	43.3 ± 1.7	42.9 ± 2.3	54.4 ± 5.7	n.s.	n.s.	n.s.
Diabetics (%)	11.2%	11.9%	10.2%	n.s.	n.s.	n.s.

Drinkers 2/2 ± 1/2 vs. 1/1, $P < 0.001$; alcohol 2/2 ± 1/2 vs. 1/1, $P < 0.01$
* $P < 0.05$
** $P < 0.01$

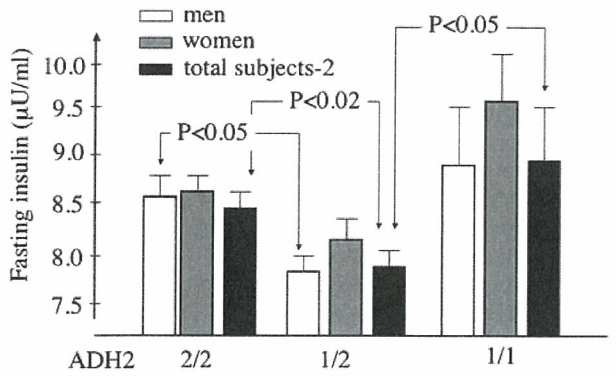

Fig. 2 Correlation of *ADH2* genotype with fasting insulin concentration in subject-2 group. Fasting insulin concentration (μU/ml): a significant difference was found between *ADH2*2/2* and *ADH2*1/2* in men (8.56 ± 0.24 vs. 7.77 ± 0.32, $P < 0.05$), and between *ADH2*2/2* and *ADH2*1/2* in total subjects-2 (8.44 ± 0.15 vs. 7.84 ± 0.20, $P < 0.02$). A significant difference was found between *ADH2*1/2* and *ADH2*1/1* in total subjects-2 (7.84 ± 0.20 vs. 8.92 ± 0.50, $P < 0.05$)

women, the *ADH2*1/2* group tended to have lower insulin concentrations (Fig. 2 and Table 4). This suggests that the *ADH2*1* allele has a lowering effect on the concentration of insulin.

Next, we compared the concentration of insulin between *ADH2*1/2* and *ADH2*1/1*. The concentration tended to be higher in the *ADH2*1/1* group than the *ADH2*1/2* group in men, women and total subjects-2, but a significant difference was only found in total subjects-2 (insulin, *ADH2*1/2*: 7.84 ± 0.20 μU/ml, *ADH2*1/1*: 8.92 ± 0.50 μU/ml, $P < 0.05$, Table 3 and Fig. 2). Because the *ADH2*1/1* group is small, the difference may have become statistically insignificant in men or in women.

In subjects-2, while the difference was statistically insignificant, the average level of HbA1c tended to be lower in the *ADH2*1/2* group than the *ADH2*1/1* or *ADH2*2/2* group (Fig. 3 and Table 4). For instance, in total subjects-2, HbA1c was 5.20 ± 0.02%, 5.17 ± 0.02%, and 5.23 ± 0.05%, respectively, in the *ADH2*2/2*, *ADH2*1/2*, and *ADH2*1/1* groups. Therefore, low insulin levels in the *ADH2*1/2* group seem to parallel low HbA1c levels, showing a U-shaped relationship with *ADH2* genotype as in Figs. 2 and 3.

Discussion

By examining the correlation between *ADH2* genotype and drinking behavior, we confirmed the previous observation that *ADH2* genotype influences the amount of alcohol consumed in a Japanese population (Higuchi et al 1996). In addition to alcohol consumption and percentage of drinkers, men from the *ADH2*1/1* group had the highest levels of AST, ALT, and γ-GTP, suggesting that they drink so much alcohol that their livers become damaged. This coincides with the observation of Tanaka et al (1996), supporting the idea that *ADH2* polymorphisms play an important role in alcoholic liver diseases.

In terms of the mechanism involved, since carriers of *ADH2*1/1* have less enzymatic activity for ethanol than carriers of *ADH2*2/1* or *ADH2*2/2*, the slow rate of ethanol clearance could damage the liver, but this is unlikely because ethanol is less toxic than acetaldehyde. Alternatively, it is possible that the slow rate of ethanol clearance protects the subjects from the uncomfortable feeling caused by acetaldehyde, thereby causing them to drink too much alcohol and leading to liver damage.

Interestingly, concentrations of insulin were higher in the *ADH2*1/1* than the *ADH2*1/2* group. Onishi et al (2003) reported that excess alcohol intake can induce insulin resistance with enhanced PI3-kinase activation. Therefore, in the *ADH2*1/1* group, excess alcohol intake may cause insulin resistance, resulting in hyperinsulinemia. Otherwise, some liver dysfunction caused by excess alcohol intake may cause a high glucose output from liver, thereby inducing hyperinsulinemia.

Table 4 Comparison of glucose–insulin axis parameters among three groups of subjects-2 divided according to the three *ADH2* genotypes

Variables				*P*-value		
ADH genotype	2/2	1/2	1/1	2/2 vs. 1/2	2/2 vs. 1/1	1/2 vs. 1/1
Men $n=$	640	346	57			
FPG (mg/dl)	103.3 ± 0.7	102.6 ± 0.9	103.3 ± 2.2	n.s.	n.s.	n.s.
HbA1c (%)	5.24 ± 0.02	5.22 ± 0.03	5.27 ± 0.08	n.s.	n.s.	n.s.
Insulin (μU/ml)	8.46 ± 0.22	7.69 ± 0.31	8.47 ± 0.75	0.0452*	n.s.	n.s.
Women $n=$	623	354	57			
FPG (mg/dl)	98.6 ± 0.6	99.3 ± 0.8	99.2 ± 2.1	n.s.	n.s.	n.s.
HbA1c (%)	5.15 ± 0.02	5.11 ± 0.03	5.17 ± 0.06	n.s.	n.s.	n.s.
Insulin (μU/ml)	8.42 ± 0.19	8.00 ± 0.26	9.36 ± 0.65	n.s.	n.s.	n.s.
Total $n=$	1,263	700	114			
FPG (mg/dl)	101.0 ± 0.46	101.0 ± 0.6	101.2 ± 1.5	n.s.	n.s.	n.s.
HbA1c (%)	5.20 ± 0.02	5.17 ± 0.02	5.23 ± 0.05	n.s.	n.s.	n.s.
Insulin (μU/ml)	8.44 ± 0.15	7.84 ± 0.20	8.92 ± 0.50	0.018*	n.s.	0.045*

*$P < 0.05$
**$P < 0.01$

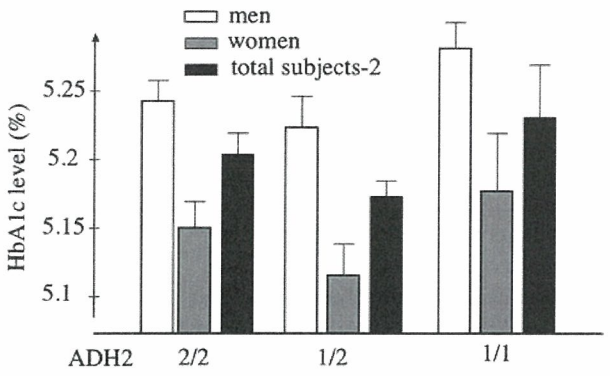

Fig. 3 Correlation of *ADH2* genotype with HbA1c level in subject-2. A significant difference was not found between the three groups. However, the HbA1c level showed a U-shaped relationship as if correlated to the insulin level

Next, we tried to focus on the *ADH2*'s genetic effects on the insulin–glucose axis. Because alcohol produces complicated effects, it is generally difficult to distinguish the genetic effects from the influence of alcohol drinking behavior. Interestingly, alcohol consumption or percentage of drinkers did not differ between the *ADH2*1/2* and *ADH2*2/2* groups (Tables 1, 2, 3 and Fig. 1a, b). This enabled us to compare the insulin concentration, dependent upon the difference in ADH2 activity itself, based on the *ADH2* polymorphism, almost independently from alcohol intake. Among subjects-2, we found that fasting insulin concentrations were significantly lower in the men and total subjects-2 with the *ADH2*1/2* genotype than those with the *ADH2*2/2* genotype (Table 4 and Fig. 2). A similar trend was seen in women, suggesting that this trend is reproducible irrespective of gender.

Thus, this study suggests that *ADH2*1* has a biphasic effect on the insulin concentration, a lowering effect with *ADH2*1/2*, and a raising effect with *ADH2*1/1* on excess alcohol intake. Interestingly, the average levels of HbA1c in subjects-2 tended to be lower in the *ADH2*1/2* group than the *ADH2*1/1* or *ADH2*2/2* groups. These two parameters seem to exhibit a U-shaped relationship (Figs. 2, 3). In nondiabetic subjects, a low insulin concentration together with a low HbA1c level usually coincides with low insulin resistance. Therefore, the above relationship suggests that light-to-moderate drinkers with the *ADH2*1* allele are likely to have reduced insulin resistance. Interestingly, this coincides with numerous other observations (Conigrave et al 2001; Hu et al 2001; Tsumura et al 1999) in terms of the notion that light drinking could benefit glucose tolerance.

Alcohol dehydrogenase catalyzed the first step in the metabolism of ethanol but has a wide range of substrates, including both aliphatic and aromatic alcohols, aldehydes, sterols, and ω-hydroxy fatty acids. We previously reported that, in the same population study, the *ADH2*1* allele is associated with increased levels of LDL-cholesterol and high blood pressure, and an increased risk of cerebral infarction (Suzuki et al 2004). The concentration of insulin or resistance to insulin could be affected by sex hormones, sex hormone-binding globulin or obesity (Falkner et al 1999; Collison et al 2000). Therefore, as another possibility, the interaction of the *ADH2*1* allele with several hormones associated with sex or lipids may decrease the insulin resistance in target tissues (Harada et al 1998).

However, in this study, the prevalence of diabetes did not differ among the three *ADH2* genotypes in subjects-1. Therefore, the effect of *ADH2* genotype on insulin resistance may be so mild or complex that it did not influence the prevalence of diabetes in the community-dwelling Japanese population. Alternatively, since all of the subjects whose FPG levels were higher than 110 mg/dl were not confirmed by the oral glucose tolerance test, if the subjects who had postprandial hyperglycemia had been included in subject-1, the result could have been different. To clarify this, a further study will be needed.

It is well known that drinking behavior is influenced more by *ALDH2* (aldehyde dehydrogenase 2) genotype than *ADH2* genotype (Higuchi et al 1996). However, although a similar investigation was performed on the correlation between *ALDH2* genotypes and their phenotype, no genetic effect of *ALDH2* was found in insulin–glucose axis and liver dysfunction (Ohsawa et al 2003). Thus, amounts of alcohol consumed would not simply depend upon insulin level.

In conclusion, this is the first paper to propose an effect of *ADH2* genotype on insulin concentrations in the Japanese. The effect seems small, although it was statistically significant due to the large number of subjects. The effect is possibly too small to have a significant bearing on the prevalence of diabetes. However, this finding provides several insights into the complex relationship between alcohol metabolism, genetic background, change in alcohol drinking behavior, the insulin–glucose axis, and the prevalence of diabetes and liver dysfunction.

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REVIEW

Contribution of somatic mutations in the mitochondrial genome to the development of cancer and tolerance against anticancer drugs

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Mitochondrial defects have long been suspected to play an important role in the development of cancer. Although most cancer cells harbor somatic mutations in mitochondrial DNA (mtDNA), the question of whether such mutations positively contribute to the development of cancer remained unclear. To clarify the role of mutant mtDNA excluding effects by the nuclear background, we focus on a method of transmitochondrial cybrids. Tumors were formed by transplanting cybrids with or without mutant mtDNA into nude mice and compared each size, revealing that mutant cybrids enhanced tumorigenesis. Next, we discuss a method for excluding the possibility of secondary nuclear mutations that may affect tumorigenesis. Mitochondrial genes that had been converted from mitochondrial to nuclear codons and equipped with a mitochondrial-targeting sequence were introduced into the nucleus of mutant cybrids. The gene products complemented the dysfunction, and reduced the promotion of tumors. By these methods, we concluded that mutant mitochondria positively and directly contribute to tumorigenesis. Since apoptosis occurred less frequently in the mutant versus wild-type cybrids in tumors, pathogenic mtDNA mutations contribute to the promotion of tumors by preventing apoptosis. Finally, we discuss the role of mutant mtDNA in conferring tolerance against anticancer drugs.
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Keywords: apoptosis; cybrid; codon; mitochondrial disease; transplantation

Introduction

The role of mitochondrial dysfunction in cancer has been a subject of great interest from various aspects. Mitochondrial defects have long been suspected to play an important role in the development of cancer. Over 50 years ago, Warburg pioneered research into the involvement of mitochondrial respiratory defects in cancer, and proposed a mechanism to explain how these defects evolve during carcinogenesis. Warburg hypothesized that a key event in carcinogenesis involved the development of an injury to the mitochondrial respiratory machinery, resulting in a compensatory increase in glycolysis, leading to lactic acidosis (Warburg, 1930, 1956). Lactic acidosis is a typical biochemical hallmark of mitochondrial diseases and is widely used in the diagnosis of mitochondrial encephalomyopathies (DiMauro *et al.*, 1985). Many groups have confirmed that the majority of cancer cells harbor somatic mutations in the mitochondrial genome which may represent the 'injury of mitochondria' described by Warburg (Polyak *et al.*, 1998; Yeh *et al.*, 2000; Liu *et al.*, 2001; Parrella *et al.*, 2001; Carew and Huang, 2002; Maximo *et al.*, 2002; Tan *et al.*, 2002). Comprehensive scanning of somatic mitochondrial DNA (mtDNA) mutations has revealed that functionally relevant point mutations in mitochondrial RNA and polypeptide-encoding genes were present in 50% of patients (Linnartz *et al.*, 2004). Thus, cancer cells seem to harbor pathogenic mutations in mtDNA as well as neutral mutations.

However, despite the close association between carcinogenesis and somatic mutations, it remained unclear whether these somatic mutations are contributors to the development of tumors or whether they simply arise as part of the secondary effects in cancer. In any case, a high frequency of mtDNA alterations in cancer and their presence in the early stages could be exploited as clinical markers for early cancer detection (Fliss *et al.*, 2000; Nishikawa *et al.*, 2001). Mitochondrial DNA alterations detected in cerebrospinal fluid may be used as sensitive markers to monitor disease progression and predict relapse (Wong *et al.*, 2003).

The high frequency of mtDNA mutations and the rapid proliferation of cancer cells with no physiological advantage has been proposed to account for the accumulation of somatic mutations in mtDNA. In fact, extensive computer modeling suggests that if a single mtDNA mutation occurs in a tumor progenitor cell, mtDNA homoplasmy (i.e., a pure population of mutant mtDNA molecules) can be achieved entirely by chance through unbiased mtDNA replication and sorting during cell division without selection for physiological advantage (Coller *et al.*, 2001). This model can explain the occurrence of neutral mutations in cancer mtDNA.

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In this review article, we will discuss a method developed to clarify the role of pathogenic mutations in mtDNA through the investigation of mitochondrial diseases. Here, we applied this method to study the contribution of mutant mtDNA in cancer by focusing on mtDNA and concluded that pathogenic mutations contribute to the promotion of tumorigenesis. Moreover, the association between mtDNA mutations and development of anticancer drug tolerance will also be addressed.

Methods that clarify contributions of mitochondrial DNA

How to investigate the role of mitochondrial DNA

The cell contains a nuclear genome and a mitochondrial genome. The human mitochondrial genome encodes two ribosomal RNA, 22 transfer RNA and 13 polypeptides. All the polypeptides are subunits belonging to the respiratory chain or the ATP synthase complex. The remaining hundreds of mitochondrial proteins are encoded by nuclear genes, synthesized in cytosol and post-translationally imported into mitochondria (Attardi and Schatz, 1988). All components involved in the maintenance, expression and regulation of the mitochondrial genetic system are encoded in nuclear genes. Thus, even though mitochondrial alterations have been described, the role of the mitochondrial genome in promoting malignant transformation is not understood. Additionally, cross-talk between these two genomes should be considered (Ohta, 2003; Nakashima-Kamimura *et al.*, 2005). Thus, when investigating the role of the mitochondrial genome, we must precisely distinguish the contribution of the mitochondrial genome from that of the nuclear genome. In particular, it would be important to understand whether many of these mutations occur during the initiation and/or development of cancer. When approaching this question, it is important to compare mtDNA in the context of identical nuclear backgrounds.

Previously, we developed a method to exclude the contribution of the nucleus to clarify the role of mutant mtDNA derived from patients with mitochondrial disorders. We constructed a hybrid cell with a common nucleus and mtDNA of interest derived from patients and could clarify that several mutations in mtDNA are conclusively responsible for the diseases. Since the cytoplasm containing mtDNA was fused with a cell lacking mtDNA, we call it a 'cybrid' instead of a 'hybrid' (Hayashi *et al.*, 1991a; Attardi *et al.*, 1995).

Establishment of cells lacking mitochondrial DNA

Before referring to cybrids, we will explain the method to completely deplete mtDNA. Since human mtDNA encodes only 13 subunits belonging to the respiratory chain or the ATP synthase complex, mammalian cells can survive without mitochondria if essential metabolic substrates are supplied. ATP can be synthesized in cytosol by glycolysis. In addition to energy metabolism, mitochondria have many other roles that are essential for living cells. Thus, mitochondria are essential for eukaryotes and cannot be excluded. However, it is possible to deplete mtDNA in cells. Indeed, yeast strains (*Saccharomyces cervisiae*) lacking mtDNA, termed $\rho 0$ are easily established when supplying fermentable carbon sources. However, yeast mitochondria could not be removed. Subsequently removal of mtDNA was also achieved in avian cells (Desjardins *et al.*, 1986). Human $\rho 0$ cells could be isolated by long exposure to ethidium bromide (EtBr), which is a strong inhibitor of mitochondrial RNA polymerase (King and Attardi, 1989: Hayashi *et al.*, 1991b). Since RNA functions as a primer for mitochondrial DNA replication, EtBr strongly inhibits DNA replication. For a long time, mouse $\rho 0$ cells could not be isolated because they became resistant to EtBr at a high frequency (Hayashi *et al.*, 1990). Instead they were isolated by long exposure with an anticancer drug (Inoue *et al.*, 1997). Human $\rho 0$ cells are cultured in a medium containing pyruvate and uridine because pyruvate is converted to lactate coupled with an oxidation of NADH to NAD^+, which is required for glycolysis and because uridine synthesis requires energized mitochondrion (King and Attardi, 1989). Currently, $\rho 0$ cells are often used as a model of a mtDNA mutation, but one should remember that it is highly possible to introduce considerable mutations into nuclear genes of $\rho 0$ cells by long exposure to EtBr. Thus, their parental cells are unsuitable for $\rho 0$ cell control.

No method for the direct introduction of mitochondrial DNA

So far, no technique to introduce full length or a fragment of mtDNA into mammalian cells has been established. Such an approach could enable to introduce a mutation of interest into a specific cancer cell to clarify the role of mutant mtDNA. On the other hand, it is possible to introduce a fragment of yeast mtDNA into yeast with a particle gun. Namely, metal particles with a fragment of mtDNA are shot mechanically into mitochondria across the plasma, and mitochondrial outer and inner membranes at high speed (Klein and Fitzpatrick-McElligott, 1993). Since yeast mitochondria have a strong recombination system, the fragment is inserted into yeast mtDNA by recombination (D'Aurelio *et al.*, 2004). However, human mitochondria have very low mtDNA recombination ability. In addition, mammalian mtDNA is easily degraded if not enveloped by mitochondrial transcriptional factor A (Tfam) (Takamatsu *et al.*, 2002); hence it is difficult to overcome the problems. A revolutionary method is yet to be established for manipulating mtDNA in human cells.

Construction of cybrids

A cybrid is constructed by fusing cytoplasm carrying mtDNA of interest with a $\rho 0$ cell (Figure 1) (Moraes *et al.*, 2001). Cytoplasm is prepared from enucleated cells (Hayashi *et al.*, 1991a); alternatively platelets or synaptosomes can be used as a cytoplasmic source (Inoue *et al.*, 1997). Since a chemical enucleating method by the exposure to actinomycin D has recently been

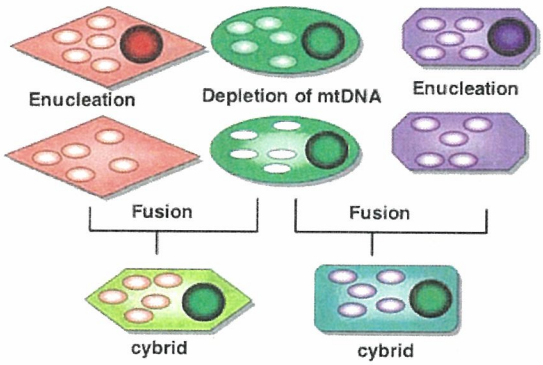

Figure 1 Construction of cybrids. Mitochondrial DNA (mtDNA) is depleted from a nucleus donor by exposure to ethidium bromide (EtBr). On the other hand, cytoplasm carrying the mtDNA of interest is prepared by removing the nucleus by treatment with cytocharasin B followed by centrifugation. Alternatively, platelets or synaptosomes can be substituted with the cytoplasm (Inoue *et al.*, 1997) or a cell treated with actinomycin D is available as a source of mtDNA (Bayona-Bafaluy *et al.*, 2003). The cytoplasm and $\rho 0$ cells were fused to construct cybrids and the resultant cybrids have a common nucleus and differ in mtDNA.

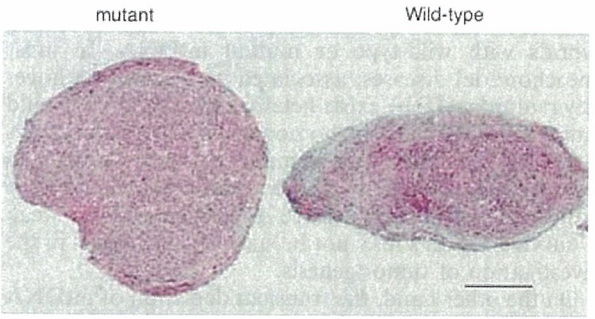

Figure 2 Tumors formed by the transplantation of mutant and wild-type cybrids. Each cybrid clone (approximately 5×10^6 cells) was injected hypodermically into nude mice to form tumors. Representative photographs of 10-day-old tumors derived from clones possessing mutant mitochondrial DNA (mtDNA) at nucleotide position 8993 (left panel) and wild-type mtDNA (right panel). Tumors derived from mutant and wild-type cybrids were stained with hematoxylin and eosin. Scale bar: 1 mm.

reported for the transfer of mtDNA to $\rho 0$ cells (Bayona-Bafaluy *et al.*, 2003), it has become easier to prepare sources of mitochondria from cancer cells.

Cybrids enabled us to confirm that mutations in mtDNA are necessarily and sufficiently responsible for decreases in mitochondrial activity. A deletion mutation was shown to be responsible for the decline in chronic progressive external ophthalmoplegia (CPEO) subgroup mitochondria (Hayashi *et al.*, 1991a). A point mutation in the $tRNA^{Lys}$ gene at nucleotide position 8344 was confirmed to be responsible for the other subgroup myoclonic epilepsy associated with ragged-red fibers (MERRF) (Chomyn *et al.*, 1991). A point mutation in the $tRNA^{Leu(UUR)}$ gene at nucleotide position 3243 or 3271 was confirmed as the cause of the mitochondrial encephalopathy, lactic acidosis and stroke-like episodes (MELAS) subgroup of mitochondrial disease (King *et al.*, 1992; Hayashi *et al.*, 1993). The cybrid method is at present widely used to determine whether a nucleotide substitution is pathogenic or the result of a simple polymorphism.

Direct contribution of mutants mitochondrial DNA revealed by the cybrid method

Transplantation of cybrids into nude mice

To explore the role of pathogenic mitochondrial mutations in the development of cancer, we applied the cybrid method with a HeLa nucleus and mtDNA mutated at nucleotide position 8993 (Holt *et al.*, 1990; Tatuch *et al.*, 1992) or position 9176 (Nakano *et al.*, 2003) in the subunit 6 gene of ATP synthase *(MATP6)*. These mutants are derived from patients with mitochondrial encephalomyopathy. However, they can be reasonably used as models of cancer mtDNA because of the reported downregulation of ATP synthase β-subunit expression in liver, kidney, colon, squamous oesophageal and lung carcinomas, as well as in breast and gastric adenocarcinomas (Cuezva *et al.*, 2002, 2004; Isidoro *et al.*, 2004). To compare mutant and wild-type cybrids in terms of their potential to form tumors, we transplanted the cybrid cells subcutaneously into nude mice. Settlement frequencies from the mutant cybrids were higher than those from the wild type. All the tumors derived from mutant cybrids were larger and grew faster than those from wild-type cybrids (Figure 2) (Shidara *et al.*, 2005). At the same time, the other group reported that cybrids with the same 8993 mutation grew to larger tumors in the same transplantation system (Petros *et al.*, 2005). We would like to emphasize that two independent groups confirmed this result by the cybrid method.

Moreover, to confirm that mutant cybrid tumors had faster growth, we transplanted a mixture (1:1) of mutant and wild-type cybrids into nude mice. The proportion of mutant mtDNA in the tumor increased progressively, and eventually, mutant mtDNA entirely replaced the wild-type mtDNA. Thus, wild-type mtDNA appeared to be selectively excluded from the mixture of wild-type and mutant cybrids. Taken together, mtDNA mutant cells have an apparent advantage to form tumors as compared to wild-type cells, providing an explanation for why homoplasmic mtDNA mutations are found in many tumors with mitochondrial dysfunction (Shidara *et al.*, 2005).

Use of cells lacking mitochondrial DNA

The transplantation of cybrids into nude mice has been employed previously to investigate the role of mtDNA in tumorigenesis. $\rho 0$ cells and cybrid cells with wild-type mtDNA were transplanted into nude mice. Tumors were formed by the cybrids containing wild-type mtDNA but not by $\rho 0$ cells (Hayashi *et al.*, 1992). In contrast, another group reported that $\rho 0$ cells could form tumors

(Morais *et al.*, 1994). However, neither study compared cybrids with wild-type or mutant mtDNA. In most mitochondrial diseases, associated with severe pathogenity mutant mtDNA exists heteroplasmically, while mild mutant mtDNA exists homoplasmically (Lightowlers *et al.*, 1997). Since most somatic mtDNA mutants in cancer reach homoplasmy, the mutations seem to have rather mild pathogenity. Since $\rho 0$ cells may be too weak to survive, $\rho 0$ cells may not be suitable as a model in the investigation of tumorigenesis.

On the other hand, the transient depletion of mtDNA influenced the expression of various nuclear genes involved in tumorigenic and invasive phenotypes (Amuthan *et al.*, 2001).

How to distinguish direct effects by mutant mitochondrial DNA from secondary nuclear mutations

Possibility of secondary mutations in nuclear genes

Since nuclear mutations as well as mtDNA mutations arise somatically at a high frequency in cancer, the potential role of mtDNA mutations in cancer development, genetic instability, and disease progression requires a careful and comprehensive investigation. Secondary mutations in nuclear genes may occur to enhance carcinogenesis during culture or transplantation after the construction of cybrids. If so, it cannot be concluded that mtDNA directly contributes to tumorigenesis, and mutant mtDNA may simply play a role as a mutagenesis of nuclear genes. Mutant mtDNA enhances the generation of reactive oxygen species (ROS), from the mitochondrial electron transport chain, that induces mutations in nuclear genes as well as mitochondrial genes. To address this issue, it would be useful to replace a mutation of mtDNA into wild type and then to examine whether potential effects on tumorigenesis are lost or remain in cybrids. However, as mentioned above, a method for manipulating mtDNA is not available. Instead of manipulating mtDNA, the import of mitochondrial proteins from cytosol into mitochondria was considered to complement mitochondrial dysfunction.

Import of mitochondrial proteins

Most mitochondrial proteins are encoded by nuclear genes, synthesized in cytosol as precursors and imported into mitochondria. Most precursor proteins are equipped with a target presequence at their N-terminus. Even if passenger proteins are not mitochondrial proteins, most of them can be imported into mitochondria when fused with a target presequence (Attardi and Schatz, 1988).

Mitochondrial genes encoding proteins are composed of mitochondrial codons. Thus, at first, codons of mitochondrial genes were converted into universal codons, and genes equipped with a target sequence gene were introduced into the nucleus and expressed in cytosol. The mitochondrial protein produced was imported into mitochondria and complemented the dysfunction derived from the mitochondrial disease (Figure 3) (Manfredi *et al.*, 2002). Additionally, the subunit 4 gene of complex I (*ND4*), a mitochondrial gene, complemented the dysfunction (Guy *et al.*, 2002). It is unknown how universally applicable this method is. Larger and/or more hydrophobic proteins may be difficult to import into mitochondria even when a target presequence is equipped to the protein.

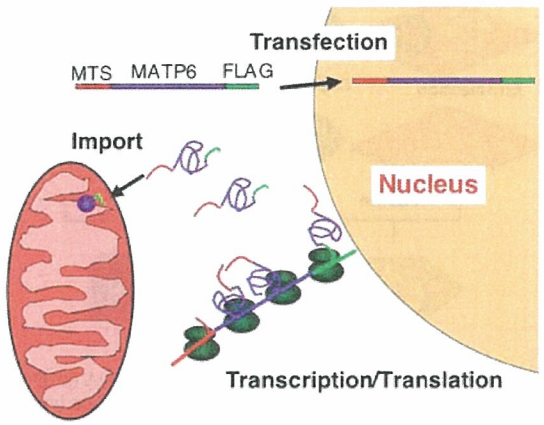

Figure 3 Expression of the mitochondrial ATP synthase subunit 6 (*MATP6*) nuclear transgene in the cytosol and its import into the mitochondria. The *MATP6* gene was converted to the nuclear version by substituting mitochondrial codons with universal codons, fused with a mitochondrial-targeting presequnce (MTS) and a FLAG tag (FLAG) for detection. The gene, wild type or mutant, was transfected into the nucleus and its product functioned in mitochondria by importing from cytosol.

Expression of a mitochondrial protein from the nucleus via cytosol

We explored whether mutant mtDNA induced secondary mutations in nuclear genes that accelerate the proliferation of mutant cybrids. As mentioned above, technology to transfect mtDNA into mammalian cells has yet to be established. Alternatively, we transfected into mutant cybrids a nuclear version of *MTATP6* whose codons had been converted into universal codons (*NuATP6*) (Shidara *et al.*, 2005). The gene contained an N-terminal presequence to target the protein to mitochondria and a FLAG tag for ease of immunodetection. We isolated stable transfectants with *NuATP6* and confirmed the expression of NuATP6 by immunostaining with anti-FLAG antibodies in cybrids carrying the mitochondrial T8993G mutation. Conversely, we transfected a nuclear version of *MTATP6* possessing the T8993G mutation (*muATP6*) into wild-type cybrids. As expected, stable transfections of *NuATP6* in mutant cybrids partially restored oxygen consumption, whereas the introduction of *muATP6* into wild-type cybrids decreased oxygen consumption. The cybrid transfectants were transplanted into nude mice and followed the tumor growth. The results indicated that the expression of functional MTATP6 slowed down tumor growth as compared to tumors derived from mock-transfected cybrids; whereas defective MTATP6 cybrids promoted

tumor growth as compared to tumors derived from mock-transfected cybrids. FLAG-positive mutant cybrids expressing wild-type MATP6 resulted in smaller tumors, indicating that functional MTATP6 conferred a disadvantage in tumor growth as compared with the expression of defective MTATP6. These results clearly show that tumorigenicity depends upon the mitochondrial function, but not upon secondary mutations in the nuclear genome.

To recapitulate *in vivo* the growth advantage conferred by muATP6 compared to wild-type cybrids, we mixed 1:1 ratio cybrid transfectants having functional MATP6 with mock ones, or conversely, cybrid transfectants having mutant MATP6 with mock transfectants, and transplanted the mixtures into nude mice. Since transfection genes were equipped with a FLAG tag, the transfectants were easily detected. Cybrids possessing mutant MATP6-FLAG became numerous in tumors. Conversely, T8,993G mutant cybrids with wild-type MATP6-FLAG were reduced in tumor promotion.

Taken together, these observations consistently showed that the growth advantage of *in vivo* tumors depends on the decline in the MATP6 function, and not upon secondary effects from the nucleus.

How to increase mutant mitochondrial DNA after a somatic mutation

Dual roles of reactive oxygen species in the development and prevention of cancer

As shown above, pathogenic mutations in mtDNA give an advantage in tumor growth and overcome wild-type mtDNA in the promotion of tumors. We will now discuss how to increase mutant mtDNA (Figure 4).

The high frequency of mtDNA alterations in cancer and their presence in the early stages of disease could be exploited as clinical markers for early cancer detection (Fliss *et al.*, 2000). ROS may be a cause for inducing the mutations. Superoxide anion radicals ($O_2^{\bullet-}$) are derived from oxygen molecules by accepting electrons that leaked from the respiratory chain. In turn, they are converted into hydrogen peroxide spontaneously or by the catalytic activity of manganese-dependent superoxide dismutase (Mn-SOD). The resulting hydrogen peroxide is then converted into a hydroxyl radical by Fenton reaction in the presence of Fe^{2+} or Cu^{+}. Hydroxyl radicals are the most reactive and toxic, possessing the potential to oxidize lipids, proteins and DNA, leading to somatic mutations. Multiple and progressive steps of mutations in genes including oncogenes and tumor suppressors are considered to contribute to neoplatsic transformation. Thus, hydroxyl radicals are a strong candidate as an inducer of cancer. Superoxide and hydrogen peroxide are sources of hydroxyl radicals, while superoxide and hydrogen peroxide play several important roles by activating many signals for regulating homeostasis (Lee *et al.*, 1998; Evans *et al.*, 2000; McCord, 2000; Michiels *et al.*, 2002). In particular, superoxide and hydrogen peroxide induce apoptosis signals (Matsuzawa *et al.*, 2005), which may protect against cancer. Thus, one must remember that ROS may have a dual role as an inducer of as well as a protector against cancer depending upon the development stage of cancer.

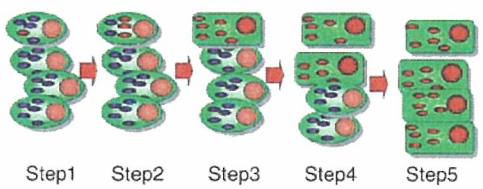

Figure 4 A model explaining the increase in mutant mitochondrial DNA (mtDNA) during the development of cancer. Step 1: A mutation occurred in an mtDNA molecule, where blue and red circles indicate wild-type mtDNA and mutant mtDNA, respectively. Step 2: Mutant mtDNA increased toward homoplasmy in a cell. Step 3: Mutant mtDNA reached homoplasmy. During the increase in mtDNA, tolerance to a low-energy condition may be achieved. Step 4: Cells with homoplasmic mutant mtDNA overcome cells with wild-type mtDNA. Since cells with mutant mtDNA are insensitive to apoptosis, cells with wild-type mtDNA relatively decrease by apoptosis. Step 5: Cells containing homoplasmic mutant mtDNA predominantly remained.

Drift of mutant mitochondrial DNA toward homoplasmy

Many mutant mtDNAs found in cancer cells exhibit homoplasmy. It is impossible to mutate all mtDNA molecules at once. Then it follows, that mutant mtDNA must overcome the wild-type DNA in a cell. Fusion between cancer cells with and without mutant mtDNA resulted in homoplasmy (Polyak *et al.*, 1998), suggesting the mutant mtDNA had overcome the wild-type in a cell. On the other hand, in the case of mutant mtDNA derived from a patient with a MELAS subgroup, heteroplasmic mutant mtDNA with a point mutation at nucleotide position 3243 was drifted toward homoplasmy in cybrids (Yoneda *et al.*, 1992). However, the results differed among cybrids with different nuclear backgrounds (Dunbar *et al.*, 1995). Thus, the behavior of mtDNA toward homoplasmy depends upon the nucleus.

When mtDNA with a long deletion was transferred into cybrids under the HeLa nucleus from a fibroblast, the deletion mutant gradually increased. However, when a galactose medium lacking glucose was supplied, cybrids with the deletion mutant increased by only a certain extent probably due to a low-energy production (Hayashi *et al.*, 1991a). In this case, the deletion mutant mtDNA may be advantageous in mtDNA replication, and a sufficient energy production by glycolysis does not affect cell growth in glucose-rich medium. On the other hand, the mutant mtDNA gives a disadvantage to cell-growth in glucose-deficient medium because sufficient energy cannot be produced by glycolysis as well as oxidative phospholylation. Thus, the balance between mtDNA replication and cell proliferation seems to determine the ratio of mutant and wild-type mtDNA. The molecular mechanism of this drift is unknown. One

possibility is a replication advantage of mutant mtDNA; alternatively, mutant mtDNA may increase by the clonal expansion of a mutant mtDNA molecule. Since the mechanism of mtDNA replication is under reconsideration (Yasukawa *et al.*, 2005), the molecular mechanism of how mutant mtDNA increases during cancer development will be understood in the near future.

Apparent increase of the growth of mutant cybrids, but no proliferation advantage

Next, we consider how mutant cells with homoplasmic mutant mtDNA overcome wild-type cells. Mutant cells with defective mitochondria should be disadvantageous in an energy production by declining oxidative phosphorylation. However, upregulation glycolytic pathway may be complementary to the increase of mutant mtDNA in a cell (Xu *et al.*, 2005). This apparent growth is faster in mutant cybrid cells than wild-type cells. To confirm the growth advantage of mutant cybrids, mutant and wild-type cybrids were mixed in a 1:1 ratio and the relative proportions of the mutant and wild-type mtDNAs were assessed in the mixed cultures. As a result, the relative content of mutant mtDNA progressively increased and eventually replaced the wild-type mtDNA.

To exclude the possibility that secondary mutations in nuclear genes led to a growth advantage in culture, the nuclear version of *MATP6* was introduced into mutant cybrids, and the resulting transfectants grew significantly slower than control mutant cybrids mock transfected with an empty plasmid. Conversely, when the mutant version was transfected into wild-type cybrids, the resulting transfectants increased significantly faster than control mock-transfected wild-type cybrids. Moreover, to confirm the effect of the transfection of the nuclear version of *MATP6* on the transfectant increase, we mixed the transfectants and mock transfectants in a 1:1 ratio. The number of *NuATP6* FLAG-positive cybrids decreased relative to the mock transfected. Conversely, there was an increase in the number of mutant FLAG-positive cybrids as compared to the mock transfectants in mixed cultures. These findings indicate that the modulation of mitochondrial ATP synthase activity via expression of mutant or wild-type MTATP6 from the nucleus can affect cell growth and override the effect of the mtDNA genotype.

There was no marked difference in Ki-67-positive cells (a proliferation marker) or in the cell cycle profiles between mutant and wild-type cybrids. Moreover, there was no difference in Ki-67-positive tumor cells derived from cybrids. These results indicate that the apparent growth advantage is not due to the enhancement of cell proliferation.

Lower frequency of apoptosis of mutant cybrids in vitro *and* in vivo

Mutant cybrids with mutant mtDNA or cybrid transfectants with the nuclear version of mutant *MATP6* exhibit alterations in energy metabolism. Then the question is why cells with an energy disadvantage increased faster. Even when the glycolytic pathway is enhanced, the decline in oxidative phosphorylation should be more predominant in energy metabolism. Nevertheless, no difference in the frequency of the Ki-67-positive cells as a proliferation index among cybrid clones was seen. Additionally, the cell cycle was not accelerated by the decline of energy production. Thus, mutant mtDNA did not confer an advantage in cell proliferation. To explore the molecular mechanism underlying the growth advantage in tumors, we hypothesized that mutant mtDNA may protect cells from apoptosis, and therefore the growth advantage of mutant cybrids may be a consequence of increased survival. To test this hypothesis, apoptosis was shown by three independent methods: terminal deoxy transferase uridine triphosphate nick end labeling (TUNEL) staining, DNA fragmentation and sub-G1 population analysed by flow cytometry. The induction of apoptosis in wild-type cybrids was seen in tumors derived from wild-type cybrids than mutant cybrids.

Relationship of apoptosis with mutant mitochondrial DNA

Apoptosis by energy crisis

Some reports indicate that mutant mtDNA induces apoptosis *in vitro* and *in vivo*, whereas other reports suggest that cells possessing mutant mtDNA become tolerant to apoptosis. Thus, the relationship between apoptosis and mutant mtDNA may be confusing. However, bioenergetic crisis in these cells may be at the core of this discrepancy. When decline in energy production reaches the minimum requirement, cells cannot survive. Several mutations derived from mitochondrial disease severely affect energy production, leading to a pro-apoptotic effect. Fibroblasts isolated from patients with a subtype of mitochondrial diseases, Leigh syndrome, undergo apoptosis in glucose-deficient medium (Geromel *et al.*, 2001), probably because neither glycolysis nor oxidative phosphorylation produces sufficient energy. The fibroblasts detached from the dish and underwent apoptosis even in glucose-rich medium. When cybrid cells carrying mutant mtDNA derived from a Leber's hereditary optic neuropathy (LHON) patient were cultured in glucose-deficient medium, they underwent apoptosis (Ghelli *et al.*, 2003). Cells with pathogenic mutant mtDNA cannot survive without a sufficient energy supply, thus apoptosis in this case seems to be a consequence of a bioenergetic crisis.

Mice with transient mtDNA depletion in various tissues were obtained by knocking out the *Tfam* gene. Apoptosis was studied in embryos with homozygous disruption of the Tfam gene and tissue-specific Tfam knockout animals exhibited a severe respiratory chain deficiency in the heart. Massive apoptosis was found in Tfam knockout embryos on embryonic day 9.5.