Diabetologia (2003) 46:1559–1566
DOI 10.1007/s00125-003-1215-4

Diabetologia

Associations between hyperglycaemia and somatic transversion mutations in mitochondrial DNA of people with diabetes mellitus

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Abstract

Aims/hypothesis. Considering that increased oxidative stress induced by hyperglycaemia plays a possible role in the pathogenesis of diabetic complications and that mitochondrial DNA (mDNA) is thought to be more vulnerable than nuclear DNA, we investigated what somatic mutations actually occur in the mDNA of diabetic patients. We also studied the relations between those mutations and urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) which is known to increase considerably in people with diabetes.
Methods. We identified somatic mutations by subcloning and sequencing two segments of mDNA [control region (nt 15996–16401) and the segment encompassing *t-RNA*$^{Leu(UUR)}$ (nt 3149–3404)] in the peripheral blood cells of six diabetic women and control subjects matched for age and sex. This was done in 20 colonies each. In each case we also assayed urinary 8-OHdG.
Results. No difference in the aggregate somatic mutational burden of mDNA was found between patients and control subjects. However, the incidence of somatic transversion mutations in mDNA was significantly higher in diabetic patients than in control subjects ($13.93 \pm 4.57 \times 10^{-5}$ vs $1.27 \pm 1.27 \times 10^{-5}$ mutations per base pair; p=0.031, according to Mann-Whitney U-test). There was no significant difference in transition mutations. A correlation was found between the transversion mutational burden and HbA_1c values, but not between it and 8-OHdG content in the urine.
Conclusions/interpretation. We showed that somatic transversion point mutations of mDNA increase in diabetic patients. Such transversion mutations can become a new biomarker for mDNA damage associated with hyperglycaemia and possibly caused by oxidative stress but not reflected by urinary 8-OHdG. [Diabetologia (2003) 46:1559–1566]

Keywords Hyperglycaemia, mitochondrial DNA, point mutations, diabetic complications, oxidative stress.

Received: 5 May 2003 / Revised: 9 July 2003
Published online: 7 October 2003
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Abbreviations: mDNA, mitochondrial DNA; nDNA, nuclear DNA; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; A, adenine; G, guanine; T, thymine; C, cytosine; ROS, reactive oxygen species.

Oxidative stress is a disturbance in the pro-oxidant [reactive oxygen species (ROS)] and antioxidant balance in favour of the pro-oxidant state [1, 2]. Increased oxidative stress has been found in people with diabetes, presumably contributing to diabetic micro- and macroangiopathic complications [3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14]. One form of oxidative damage to cellular macromolecules, mutations in mitochondrial DNA (mDNA) have been noted as a possible consequence of DNA damage [13, 15, 16]. Most experiments on diabetes mellitus have been directed to large mDNA deletion [13, 15], and experiments on somatic point mutations have been very limited [16]. Somatic point mutations have also been investigated in con-

nection with aging or age-related diseases [17, 18, 19, 20].

MDNA has a higher frequency of somatic mutations than nuclear DNA (nDNA). Deamination and methylation are thought to bring about transition mutations, i.e. the substitution of one purine or one pyrimidine base for its respective other, in nDNA and mDNA [21, 22, 23]. Both processes often occur in vivo [24]. Transversion mutations occur in the same way through oxidative damage caused by 8-hydroxy-2′-deoxyguanosine (8-OHdG) [25, 26, 27, 28, 29, 30]. Along with these mutations, mitochondria are also likely to have fewer repair enzymes, e.g. glycosylase, for base excision repair than the nucleus has [31], and no nucleotide excision repair capacities [32]. MDNA is also located near the respiratory chain, potentially generating ROS. In addition, mDNA has no histone proteins, which protect DNA from deamination, methylation or oxidation [23, 33]. As a result, mDNA is thought to have a higher mutability and subsequently acquire 5 to 20 times as many somatic mutations as nDNA [34, 35].

Because of its abundance and mutagenicity 8-OHdG has been reported to be the most important indicator of oxidative DNA damage [5, 25, 36]. This 8-OHdG comes from an oxidative reaction to the guanine (G) base, which is highly susceptible to oxidative stress, since it has the lowest oxidation potential among bases. The substance 8-OHdG promotes the formation of Watson-Crick pairs not only with cytosine (C), but also with adenine (A), and can give rise to G:C site to thymine (T):A site or T:A site to G:C site transversion point mutations (substitution of a purine base for a pyrimidine base or vice-versa; referred to below as “8-OHdG-associated mutations”) [25, 37, 38]. The amount of 8-OHdG is reportedly larger in the DNA and urine of people with diabetes than in those of non-diabetic people [5, 7, 9]. Urinary 8-OHdG is a familiar marker of oxidative stress, because it is quite stable in the urine and not easily affected by foods [39, 40].

We therefore attempted to identify the kinds of mutations that can actually be induced in the mDNA of patients with diabetes mellitus, while concurrently assaying urinary 8-OHdG.

Subjects and methods

Subjects. Patients were recruited from the outpatient department of the Shinshu University Hospital. For the control group non-diabetic subjects of the same sex and age were selected at random. All participants gave their written informed consent before enrolling in the study, which was approved by the Ethics Committee of Shinshu University School of Medicine, Matsumoto, Japan. The type of diabetes was diagnosed according to the 1998 criteria of the World Health Organization [41]. Diabetic retinopathy diagnosed by funduscopy was designated as a marker of microangiopathy. One patient had clinically overt macroangiopathy (cerebral infarction). There were no active smokers among our subjects, whose characteristics are shown in Table 1. Postprandial peripheral blood and urine samples were taken from each subject. The blood samples were subjected to DNA extraction and urine samples were frozen at −20°C until analysis.

DNA extraction and subcloning. DNA was extracted from peripheral blood cells using a DNA Extractor WB kit (NaI method; Wako Pure Chemical Industries, Osaka, Japan). The extracted DNA (0.5 to 1.0 μg) was subjected to a PCR with Taq DNA polymerase (Takara Bio Shiga, Japan) to amplify two segments of mDNA (GenBank J01415), nt 15997 to 16400 (forward primer, 5′-CCA CCA TTA GCA CCC AAA GC-3′: backward primer, 5′-TGA TTT CAC GGA GGA TGG TG-3′), and nt 3150 to 3403 (forward primer, 5′-AGG ACA AGA GAA ATA AGG CC-3′: backward primer, 5′-CAC GTT GGG GCC TTT GCG TA-3′). The former (segment A) is a hypervariable segment, the latter (segment B) is a segment encompassing the *t-RNA*$^{Leu (UUR)}$ gene. PCR was done in only 12 cycles consisting of denaturation at 94°C, annealing at 55°C and extension at 72°C for 1 min each. The PCR products were electrophoresed on a 1.5% (wt/vol) agarose gel with a Ready-to-Load 100 bp ladder (Invitrogen, Carlsbad, Calif., USA) running in parallel for approximate PCR product band sizing and stained by ethidium bromide for 15 min. Proper bands at the size of 444 bps (segment A) or 294 bps (segment B) were then cut out, and the PCR products were purified by a GFX PCR

Table 1. Characteristics of diabetic and non-diabetic control subjects

Diabetic patients							Control Subjects			
Patient no. (*n*=6)	Age (years)/ sex	Type of diabetes	Duration of diabetes (years)	Treatment	Complications	HbA_1c (%)	Control no. (*n*=6)	Age (years)/ sex	HbA_1c [%]	Comments
DM_1	35/F	Type 1	9	Insulin	None	8.0	Cont_1	37/F	5.1	None
DM_2	47/F	Type 2	2	OHA	None	7.5	Cont_2	46/F	5.0	None
DM_3	53/F	Others*	29	Insulin	Micro	10.2	Cont_3	52/F	5.2	None
DM_4	63/F	Type 1	32	Insulin	Micro	8.0	Cont_4	63/F	5.0	Hyperlipidaemia
DM_5	66/F	Type 2	2	Insulin	None	8.2	Cont_5	64/F	5.0	None
DM_6	68/F	Type 2	20	Insulin	Micro/Macro	9.4	Cont_6	71/F	5.3	Hypertension

*This patient had 2/20 A3243G point mutations, which were excluded as germ-line mutations in heteroplasmy.

DM_1, 2 etc., diabetic patient no. 1, 2 etc.; Cont_1, 2 etc., control subject no. 1, 2 etc.; OHA, oral hypoglycaemic agent; Micro, microangiopathy; Macro, macroangiopathy

DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech, Piscataway, N.J., USA). The purified PCR products were ligated into a pCR2.1 vector and transformed into competent cells of *Escherichia coli* by using an Original TA cloning Kit (Invitrogen). The transformed cells were then spread onto LB agar plates containing 143.1 μmol/l of ampicillin with 40 μl of 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) stock solution (48.9 mmol/l).

Sequencing of mDNA. Recombinant plasmids isolated from 20 white colonies per patient or control subject were subjected to sequencing with a dye terminator cycle sequencing kit (Perkin-Elmer, Foster, Calif., USA). The array of mDNA sequences was determined by using the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster, Calif., USA) and comparing carefully with the revised Cambridge Reference Sequence [42]. The mutations identified in all clones (homoplasmy) are referred to as "germ-line mutations" and the other heteroplasmic mutations as "somatic mutations". The two A3243G transition mutations from 20 colonies in patient DM_3 (Table 1) were the only exceptions. They were qualified as germ-line mutations in heteroplasmy, because her daughter had been found to have mitochondrial diabetes.

Urinary 8-OHdG assay. The 8-OHdG in the urine was measured by enzyme-linked immunosorbent assay [43]. The intra- and inter-assay coefficients of variation were 6.0% and 8.6% respectively. We calculated the urinary 8-OHdG by dividing 8-OHdG by creatinine per unit volume of urine.

HL-60 cell culture. The HL-60 human promyelocytic/myeloblastic cell line was purchased from the American Type Culture Collection (Rockville, Md., USA) and maintained in a RPMI 1640 medium (11.1 mmol/l glucose; Nikken BioMedical Laboratory, Kyoto, Japan), supplemented with 10% fetal bovine serum (Life Technologies, Grand Island, N.Y., USA) and 1% antibiotic-antimycotic (100 U/ml penicillin G sodium, 171.9 μmol/l streptomycin and 270.5 nmol/l amphotericin B; Invitrogen). The HL60 cells were divided into two groups, which were further cultured with glucose concentrations of 5.6 and 27.8 mmol/l respectively, by adding phosphated-buffered saline and/or a concentrated glucose solution. After 6 weeks of incubation, somatic point mutations in mDNA were assessed as described earlier.

Mathematical modelling of PCR error. Mathematical modelling of the PCR error [18] was as follows: Let f be the PCR polymerase error rate per base, L the length of the strand to be amplified, S_0 the initial number of strands and η the fraction of molecules replicated in each cycle. The probability of introducing k errors while copying a strand is:

$$p(k) = C(L,k) f^k (1-f)^{L-k}.$$

Let $S(c,k)$ be the number of strands present after c cycles with k PCR-induced errors. The fraction of strands present after c cycles with k errors is:

$$\begin{aligned} F(c,k) &= S(c,k)/(S_0(1+\eta)^c) \\ &= [1/(1+\eta)]\Big[F(c-1,k) \\ &\quad + \eta \textstyle\sum_{j \leqslant k} F(c-1,j)\, p(k-j)\Big]. \end{aligned}$$

Therefore, the average number of errors per strand is:

$$< k >= \textstyle\sum_k F(c,k)k = cfL\eta/(1+\eta).$$

The overall PCR error per base is:

$$< k > /L = cf\eta/(1+\eta).$$

Data analysis. The mutational burden for a given sample was calculated as the number of mutations found divided by the total number of bases examined. The data were reported as the mean ± SEM. The correlation between variables was calculated by the linear regression analysis of untransformed values. A p value lower than 0.05 was regarded as representing a statistically significant difference. The Mann-Whitney U-test was used to compare quantitive data from two segments of mDNA and two groups. Fisher's exact test was used to compare the ratios of the different kinds of somatic transition mutations. All analyses were done using Statview 5.0J (Abacus Concepts, Berkeley, Calif., USA).

Results

The PCR error per base calculated during 12 PCR cycles was ~6.00×10^{-4}/bp, with an error rate of ~10^{-4} per base. To test the overall PCR error per base, we experimentally measured PCR errors by using a single bacterial colony as a template for PCR. Sequencing of 25 recombinant plasmids for segment A and 32 for segment B revealed no point mutations, indicating that the overall PCR error per base in the 12 PCR cycles was less than 5.49×10^{-5}/bp. When all the molecules had been replicated during each cycle, the PCR error rate was less than 9.15×10^{-6}/bp.

The frequency of germ-line mutations (polymorphisms) was significantly higher in segment A than in segment B ($49.51 \pm 5.90 \times 10^{-5}$ mutations per bp for A, $1.79 \pm 1.79 \times 10^{-5}$ mutations per bp for B; $p<0.0001$, according to Mann-Whitney U-test). However, no differences were found between segments A and B in the frequency of (i) total somatic mutations ($44.35 \pm 9.57 \times 10^{-5}$ mutations per bp and $59.06 \pm 10.94 \times 10^{-5}$ mutations per bp; Table 2), (ii) transversion mutations ($6.19 \pm 3.23 \times 10^{-5}$ and $10.74 \pm 5.55 \times 10^{-5}$), (iii) transition mutations ($37.13 \pm 10.22 \times 10^{-5}$ and $44.74 \pm 9.23 \times 10^{-5}$), and (iv) insertions and deletions ($1.03 \pm 1.03 \times 10^{-5}$ and $3.58 \pm 2.40 \times 10^{-5}$).

Since there were no differences in the mutational burden between the two segments, we assessed the aggregate somatic mutational burden by combining segments A and B. None of the somatic mutations detected in the twenty clones were the same, suggesting that the degree of heteroplasmy was less than 5%. There were no significant differences in the total somatic point mutational burden between the diabetic and control group ($49.39 \pm 8.50 \times 10^{-5}$ mutations per bp in patients and $51.93 \pm 12.00 \times 10^{-5}$ mutations per bp in healthy control subjects; $p=0.58$).

However, the aggregate somatic transversion mutational burden in diabetic patients was significantly higher than in the control subjects ($13.93 \pm 4.57 \times 10^{-5}$ mutations per bp vs $1.27 \pm 1.27 \times 10^{-5}$ mutations per bp; $p=0.031$; Fig. 1a), while no significant differences were found when two segments were analysed separately ($p=0.30$ and 0.068, in segments A and B respectively). The frequency of somatic transition point mutations was not different between the two groups

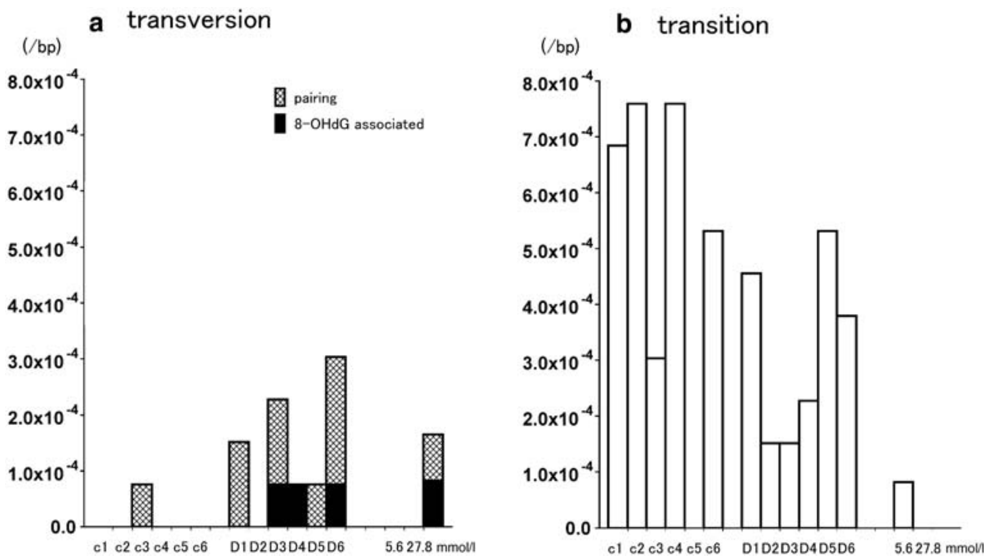

Fig. 1a, b. Aggregate incidence of somatic mutations in leucocyte mDNA from control subjects (c1–c6), diabetic patients (D1–D6) and HL-60 grown for 3 weeks at glucose concentrations of 5.6 and 27.8 mmol/l. The aggregate transversion mutational burden (number of transversion mutations found in multiple clones, divided by the total number of bps sequenced per subject) is shown (**a**) for each of six samples from control subjects and from patients with diabetes, and for one sample of HL-60 per glucose concentration. The black bar represents 8-OHdG-associated transversion mutation. The double-hatched bar represents the other transversion mutations between Watson-Crick base pair; like A:T site to T:A site, or G:C site to C:G site, or vice-versa. The aggregate transition mutational burden (number of transition mutations found in multiple clones, divided by the total number of bps sequenced per subject) is shown in a similar way (**b**)

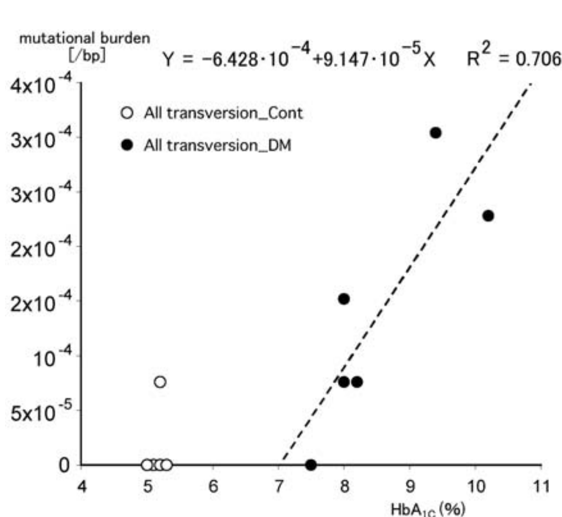

Fig. 2. HbA_1c values and somatic transversion mutational burden of mDNA in peripheral blood cells from control subjects and diabetic patients. The white circles represent total transversion in control subjects. The black circles represent total transversion in diabetic subjects. Among diabetic patients there is a positive correlation between the incidence of somatic transversion mutations and HbA_1c values (r=0.840, r^2 =0.706, p=0.036; Y=−6.428*10−4+9.147*10−5X, according to linear regression analysis)

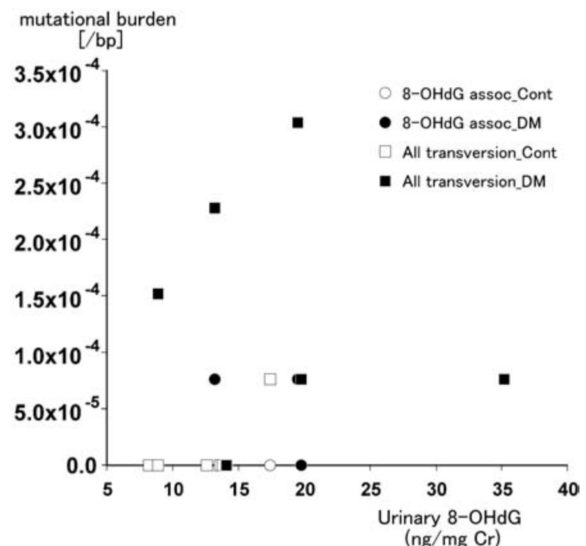

Fig. 3. Urinary 8-OHdG and somatic transversion mutational burden in leucocyte mDNA from control subjects and diabetic patients. Including all samples from all subjects, there is a negative correlation between the total number of transversion mutations, 8-OHdG-associated transversion mutations and urinary 8-OHdG. The white square represents total transversion in control subjects. The black square represents total transversion in diabetic subjects. 8-OHdG-associated transversion mutations in control subjects are depicted by white circles. In diabetic patients they are depicted by black circles

($31.66 \pm 6.63 \times 10^{-5}$ vs $50.65 \pm 12.35 \times 10^{-5}$; p=0.17; Fig. 1b). Interestingly, insertions (1) and deletions (2) were detected in only three diabetic patients. In addition, the linear regression analysis showed a significant correlation between aggregate somatic transversion mutational burden and HbA_1c values in diabetic patients (r=0.840, r^2 =0.706, p=0.036; Fig. 2). The HbA_1c values were 8.55±1.03% in patients with dia-

Table 2. All somatic mDNA mutations and urinary 8-OHdG

Name	Urinary 8-OHdG [ng/mg Cr]	Segment	Somatic point mutations		
			Transversion mutations	Transition mutations	Insertions and deletions
Cont_1	8.20	A	None	T16024C,T16029C,T16123C,A16200G,A16293G	None
		B	None	A3156G,T3206C,A3307G,T3344C	None
Cont_2	13.60	A	None	T16018C,G16060A,A16216G,C16286T,C16295T,T16325C,T16356C,T16381C	None
		B	None	A3268G,T3290C	None
Cont_3	17.41	A	T16020A	T16086C	None
		B	None	T3272C,T3321C,T3336C	None
Cont_4	12.65	A	None	T16024C,A16037G,C16076T,C16079T,A16194G,A16219G,A16293G,C16327T	None
		B	None	T3250C,C3310T	None
Cont_5	8.92	A	None	None	None
		B	None	None	None
Cont_6	13.79	A	None	A16037G	None
		B	None	T3150C,T3181C,T3265C,A3276G,A3289G,T3371C	None
DM_1	8.90	A	T16022A	C16193T,C16225T,C16267T	None
		B	A3180T	T3323C,A3348G,T3350C	None
DM_2	14.12	A	None	None	None
		B	None	G3316A,C3317T	None
DM_3	13.20	A	***A16120C***,A16293T,T16330A	G16308A	None
		B	None	A3262G	3227–9 del T
DM_4	35.20	A	None	A16177G,A16289G	16184–16188 ins C
		B	***T3237G***	T3174C	None
DM_5	19.80	A	None	A16098G,C16239T,A16265G,T16341C,C16378T	None
		B	T3378A	C3162T,T3386C	3273 del T
DM_6	19.53	A	C16358G	A16077G,C16321T	None
		B	***A3217C***,T3220A,A3233T	A3267G,A3367G,T3386C	None

8-OHdG-associated mutations are in bold, italic letters.
DM_1, 2 etc., diabetic patient no. 1, 2 etc.; Cont_1, 2 etc., control subject no. 1, 2 etc.; Cr, creatinine

betes mellitus and 5.13±0.12% in the control subjects (p<0.005). Moreover, two transversion mutations including one "8-OHdG-associated mutation" were detected from 30 clones of mDNA from HL60 cells cultured at a glucose concentration of 27.8 mmol/l. At a glucose concentration of 5.6 mmol/l one transition mutation was found (Fig. 1a, b).

The amount of 8-OHdG in the urine tended to be slightly larger in diabetic patients than in control subjects (18.46±3.75 ng/mg creatinine compared with 12.43±1.39 ng/mg creatinine). Urinary 8-OHdG was not correlated with the amount of transversion or transition point mutations of mDNA in any of the subjects (Fig. 3). Also no relation was found between age and HbA_1c values. We only observed 8-OHdG-associated transversion mutations in three patients with diabetes mellitus, and at 22.64±6.54 ng/mg creatinine their average concentration of urinary 8-OHdG was slightly higher than in the other diabetic patients.

Discussion

By means of a method similar to one already used [18], we directly assessed the aggregate burden of somatic point mutations in two segments of mDNA by using a PCR-cloning-sequencing strategy to detect acquired somatic point mutations. In our study it was necessary to take the PCR error into account. Taq DNA polymerase has an assumed error rate of $\sim 10^{-4}$ per base per doubling, resulting mainly in A:T to G:C transition mutations. Other artefacts such as heat-induced mutagenesis can also be created during PCR [44]. To keep the PCR error per base at a minimum, we used only 12 PCR cycles. According to the mathematical formula [18], the artificial mutational burden during 12 cycles of PCR with an error rate of $\sim 10^{-4}$ per base per doubling was $\sim 6.00\times 10^{-4}$/bp. However, the calculated artificial mutational burden was much higher than the 5.49×10^{-5}/bp which we obtained by the experimental assessment of PCR error per base. This indicates that under the conditions of our experiment the true error rate of Taq DNA polymerase was lower than expected. Our method was designed to systematically detect precise somatic point mutations even when the degree of heteroplasmy was low. Therefore we believe that our method was particularly effective for studying somatic mutations.

The control region (D-loop) is known to be the most variable portion of the mDNA genome [17]. Our estimated aggregate somatic point mutational burden was of a similar size to the aggregate mutational burden found in the D-loop of two elderly persons ($35\sim 90\times 10^{-5}$) [45] and in brain mDNA ($17.5\pm 15.9\times 10^{-5}$ mutations per bp in all elderly subjects) [18]. As in our study, somatic transition mutations were also shown to be a major part of total somatic mutations [18, 45].

With regard to the reported somatic transition mutations caused by oxidation, oxidised C has been reported to cause C to T transition mutations from oxidative stress in *E. coli* [22]. These C to T or G to A transition mutations accounted for only a small percent of the somatic transition mutations detected in our study (10/25 in diabetic patients and 7/40 in control subjects, p=0.17 according to Fisher's exact test). There were also no mutual relations between these transition mutations, HbA_1c values and urinary 8-OHdG. Other studies have also used peripheral blood cells to examine the accumulation of A3243G mutations in mDNA of diabetic patients, quantifying its mutational burden as $5.2\pm 0.3\times 10^{-5}$ mutations per bp [16]. Even allowing for the PCR error ($\sim 5.49\times 10^{-5}$/bp), this value is much lower than ours ($31.66\pm 6.63\times 10^{-5}$/bp). Hence, it is possible that nt 3,243 is not a hot point for mutation as stated in that report.

Concerning the correlation we found between the incidence of somatic transversion mutations in mDNA and HbA_1c values, many reports have dealt with somatic mutations in mDNA. Most of them, however, have been on somatic transition mutations, deletions and insertions, whereas somatic transversion mutations have never been reported. In addition, only few documents exist on somatic point mutations associated with hyperglycaemia or HbA_1c values. Our in vitro experiment using HL60 cells supported our findings on somatic transversion mutations in vivo.

In our study 8-OHdG in the urine of the diabetic patients was only a little higher than that in control subjects, although previous papers [7, 9, 13] showed considerably higher concentrations of urinary 8-OHdG in patients with diabetes mellitus. In addition, no correlation was found between urinary 8-OHdG and the transversion mutational burden. However, oxidative stress is reported to be increased in people with diabetes mellitus or hyperglycaemia [3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15], suggesting that the somatic transversion mutational burden of mDNA is associated with oxidative stress derived from hyperglycaemia, which is not reflected by urinary 8-OHdG.

Concentrations of urinary 8-OHdG in three diabetic patients who had microangiopathy or macroangiopathy or both, and also had 8-OHdG-associated mutations, were much higher than in the other subjects. These findings confirm the conventional idea [5, 9, 13, 15] that 8-OHdG is connected with diabetic complications. Mutations associated with 8-OHdG have not previously been shown in patients with diabetic complications. However, our study only detected three 8-OHdG-associated mutations, which did not correlate with HbA_1c values.

It is possible that somatic mutations associated with hyperglycaemia occur not only in mDNA, but also in nDNA in very small amounts. In the light of many reports on diabetic complications in association

with hyperglycaemia or HbA_1c values [46, 47, 48], it is possible that such somatic transversion mutations also partly contribute to diabetic complications. Alternatively, common mechanisms could result in the complications and the somatic transversion mutations associated with hyperglycaemia. In conclusion, the increase detected by us in somatic transversion point mutations in mDNA from diabetic patients could be used as a novel biomarker.

Acknowledgements. We extend special thanks to: Dr S. Ohta (Department of Preventive Medicine, Shinshu University School of Medicine, Matsumoto, Japan) for her useful advice about statistics; Dr D.P. Cioca (2nd Department of Internal Medicine, Shinshu University School of Medicine) for a generous gift of HL-60 cells; Mr. and Mrs. Junker for language assistance; Professor K. Kiyosawa (2nd Department of Internal Medicine, Shinshu University School of Medicine) for his helpful suggestions.

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