検例であり、男女比は 1.1:1、平均年齢は 80 歳である。MTHFR 遺伝子の codon677 における non-synonymous SNP(C→T ; alanine→valine)をタイピングし、JG-SNP に登録された 34 種の悪性腫瘍との関連を検討した。

B. 結果

JG-SNP に登録された 34 種の悪性腫瘍のうち、症例数が 10 を超えるものは、肺癌(腺癌) (64)、肺癌 (扁平上皮癌) (41)、肺癌 (小細胞癌) (37)、骨髄性白血病 (88)、骨髄腫 (21)、悪性リンパ腫 (65)、胃癌 (164)、食道癌 (17)、結腸癌 (101)、直腸癌 (31)、膵癌 (48)、肝細胞癌 (49)、胆道癌 (48)、前立腺癌 (85)、乳癌 (64)、子宮癌 (15)、腎癌 (21)、尿路癌 (26)、甲状腺癌 (25)、頭頸癌 (11) であり、連関解析はこれらの悪性腫瘍について行った。これらの悪性腫瘍のうち、minor allele(677T)をヘテロまたはホモに有する群が minor allele を持たない群に対して、有意な相対危険率を見いだされた悪性腫瘍は、直腸癌(相対危険率 0.27, 95%信頼区間; 0.12−0.60, p=0.001)と、悪性リンパ腫(相対危険率; 1.88, 95%信頼区間; 1.03−3.43, p=0.039)であった。

C.考察

直腸癌との関連はこれまでの報告を裏付けるものである。悪性リンパ腫との関連については従来から controversial であり、さらなる検討を要する。今回の結果は近年増加の一歩をたどる悪性腫瘍の一部に対して、中高年層における葉酸やビタミンＢ１２の補充の有効性を示唆する有用な情報であると考えられる。

結論：MTHFR 遺伝子多型性(677C>T) と多種類の悪性腫瘍との関連を単一のデータベース(JG-SNP) 上で初めて検討した結果、有意な関連が、直腸癌と悪性リンパ腫との間で認められた。

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厚生労働科学研究費補助金　(長寿科学研究)
分担研究報告書
老人性疾患と遺伝子多型
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研究要旨：
高齢者疾患の予防と治療の質を向上し効率的に行うためには、疾患発症における遺伝子と環境因子の相互作用を明らかにすることは重要である。本研究は、東京都老人医療センターの連続剖検例を用いて、老年病に関連する候補遺伝子の一塩基多型(SNP)の関連を網羅的に解析して、データベース化し、広く医学研究ならびに医療の進歩に寄与することを目的とする。

A.　研究目的

高齢化社会の本格的な到来を迎えて、老人性疾患の３大死因である、循環器疾患、脳血管疾患および癌を制御することが重要な課題となってきている。本研究では、「老年病ＳＮＰデータベース」(略称　JG-SNP) のに登録された病理解剖材料を用いて、これに遺伝子解析を施行し、老人性疾患発症の遺伝子および環境因子の相互作用をあきらかにすることである。また、その成果をデータベース化して、今後の老年病研究に資することを目的とする。

B.　研究方法

用いた「老年病ＳＮＰデータベース」(略称JG-SNP)は病理解剖材料を用いて解析された臨床病理学的表現型をもつことが特徴的であり、以下の利点を持つ。1. 病理診断は臨床診断に比べてはるかに高精度の診断が可能である。2. 病理解剖では全身の臓器を採取し、くまなく検索するため臨床診断のついていない、例えば潜伏癌の症例が多数含まれている。3. 平均年齢が80 歳とほぼ平均寿命に匹敵する高齢者集団である。従って超高齢者でのみ発症する一部の老年病を除いて、ほとんどの症例では生涯で発症しうる可能性のある病気はほとんど発症していると推定される。4. 90 歳から 100 歳以上の対象例を多数含み、長寿に関与する遺伝子の同定が可能である。以上の老年病 SNP データベースに登録してある高齢者剖検例約 1500 例を対象として SNP の解析を行った.

B.　研究結果

SNP 解析は融解曲線分析法にて行い、合計４４遺伝子、６７SNP を解析を終了した。遺伝子／SNP は既報の論文、データベースなどから、老人性疾患との関連が示唆されているものを候補として選択した。多くの SNP はハーディーワインバーグ平衡を満たしており、日本人の標準 SNP 頻度に近似していた(JSNP データベース)。疾患については、老年２６疾患に関する病理診断および臨床診断を、動脈硬化に関しては、病理的動脈硬化指数(PAI)を目的変数とし、これと相関する遺伝子多型を多変量解析を用いて検討した。その結果、各疾患および動脈硬化レベルと相関する遺伝子多型が複数抽出された。

C.　考察

老年病 SNP データーベースの網羅的相関解析はまだ進行中であるが、その成果として医科のものが考えられる。1. 老化、老年病関連遺伝子の SNP 頻度情報を得ることが出来る。2. 老化、老年病関連遺伝子の SNP に対して老年病との相関解析（患者- 対照研究と呼ばれる）が可能である。特定の疾患に関して逆に登録済みの老年病関連遺伝子の SNP 頻度を知ることが出来る。3. 特定の老年病と候補遺伝子 SNP との関連が特定できればその病因、発生病理の解明につながり、ひいては老年病の予防が可能となる。また、個人の有する遺伝子多型による疾患の起こりやすさ、治療に対する反応性を予想することが可能になりオーダーメイド医療・創薬の分野でも役立つ。

D. 結論

今回の老年病 SNP データーベースを拡充する取り組みにより、老人性疾患と遺伝子および環境因子の相互作用を解析するための基盤ができあがった。

E. 健康危険情報

なし

F. 研究発表（論文発表）

なし

H. 知的財産権の出願・登録状況

特許取得：なし

実用新案登録：なし

研究成果の刊行に関する一覧表

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coq7/clk-1 regulates mitochondrial respiration and the generation of reactive oxygen species via coenzyme Q

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Summary

***coq7/clk-1* was isolated from a long-lived mutant of *Caenorhabditis elegans*, and shows sluggish behaviours and an extended lifespan. In *C. elegans* and *Saccharomyces cerevisiae*, *coq7/clk-1* is required for the biosynthesis of coenzyme Q (CoQ), an essential co-factor in mitochondrial respiration. The *clk-1* mutant contains dietary CoQ_8 from *Escherichia coli* and demethoxyubiquinone 9 (DMQ9) instead of CoQ_9. In a previous study, we generated COQ7-deficient mice by targeted disruption of the *coq7* gene and reported that mouse *coq7/clk-1* is also essential for CoQ synthesis, maintenance of mitochondrial integrity and neurogenesis. In the present study, we rescued COQ7-deficient mice from embryonic lethality and established a mouse model with decreased CoQ level by transgene expression of COQ7/CLK-1. A biochemical analysis showed a concomitant decrease in CoQ_9, mitochondrial respiratory enzyme activity and the generation of reactive oxygen species (ROS) in the mitochondria of CoQ-insufficient mice. This implied that the depressed activity of respiratory enzymes and the depressed production of ROS may play a physiological role in the control of lifespan in mammalian species and of *C. elegans*.**

Key words: cog7/clk-1, coenzyme Q, mitochondrial respiration, reactive oxygen species.

Introduction

clk-1 is one of the longevity mutants discovered in *Caenorhabditis elegans*. This mutant shows an extended lifespan, delayed embryonic and postnatal development, and slow behaviours such as retarded defecation, swimming and pharyngeal pumping (Wong *et al.*, 1995; Lakowski & Hekimi, 1996). The primary sequence of the causal gene, *clk-1*, is conserved through evolution among various species ranging from yeast to mammals (Asaumi *et al.*, 1999; Ewbank *et al.*, 1997; Vajo *et al.*, 1999). Sequence homology showed that *clk-1* is the mammalian orthologue of yeast *coq7/cat5* and this plays an essential role in the biosynthesis of coenzyme Q (ubiquinone, CoQ) (Fig. 1) (Marbois & Clarke, 1996). CoQ transfers electrons from complex I to complex III, or complex II to complex III, in the mitochondrial inner membrane (Do *et al.*, 1996). Previous studies demonstrated that the majority of superoxide ($O_2^{\bullet -}$) is generated at the CoQ site in the mitochondrial respiratory chain (Lass *et al.*, 1997). Reactive oxygen species (ROS) such as $O_2^{\bullet -}$, $OH^{\bullet}$, H_2O_2 and $ONOO^{\bullet -}$ are endogenous substances harmful to macromolecules such as DNA, lipids and proteins, damage to which eventually limits the lifespan of animals (Sohal & Weindruch, 1996). CoQ presents the characteristic structure of a redox-active quinone with a variable number of isoprene units, ranging from seven to 12 depending on the animal species. For example, in *C. elegans* and rodents, CoQ_9 is the dominant form, whereas CoQ_{10} is the dominant form in other mammals such as bovines and humans (Lass *et al.*, 1997; Jonassen *et al.* 2001) showed that *clk-1* mutant worms failed to produce endogenous CoQ_9, but instead absorbed exogenous CoQ_8 from dietary *E. coli Clk-1* mutant worms, and then accumulated demethoxyubiquinone 9 (DMQ_9), the precursor of CoQ_9, because the mutants cannot catalyse the conversion from DMQ_9 to CoQ_9 (Fig. 1). Therefore, it is not surprising that the *clk-1* mutant fails to develop when dietary CoQ_8 was removed by supplying them with CoQ-deficient *E. coli* (Jonassen *et al.*, 2001). In this context, the deficiency of CoQ_9 confers extended lifespan to *C. elegans* under conditions where the dietary CoQ_8 is just sufficient for the worm's development and survival. It is interesting, then, to note that COQ7-deficient mice, which exhibited an abnormal accumulation of DMQ_9, fail to grow beyond embryonic day 10.5 (Nakai *et al.*, 2001). Overall, the data provide only limited support for the possibility that DMQ_9 functions as an electron messenger to support the development and survival in *C. elegans* and mice. Conversely, wild-type *C. elegans* showed an extended lifespan when dietary CoQ_8 was removed (Larsen & Clarke, 2002). Therefore, a reduced level of CoQ in mitochondria may extend the lifespan of *C. elegans*.

In this study, we successfully rescued COQ7-deficient mice from embryonic lethality by exogenous expression of *clk-1* in transgenic mice. Interestingly, the rescued COQ7-deficient mice showed a decreased level of CoQ in the kidney, and we further showed a significant correlation between the mitochondrial enzyme activities and ROS production in the model mice. The results imply that this model system may shed light on the biochemistry of lifespan in mammals.

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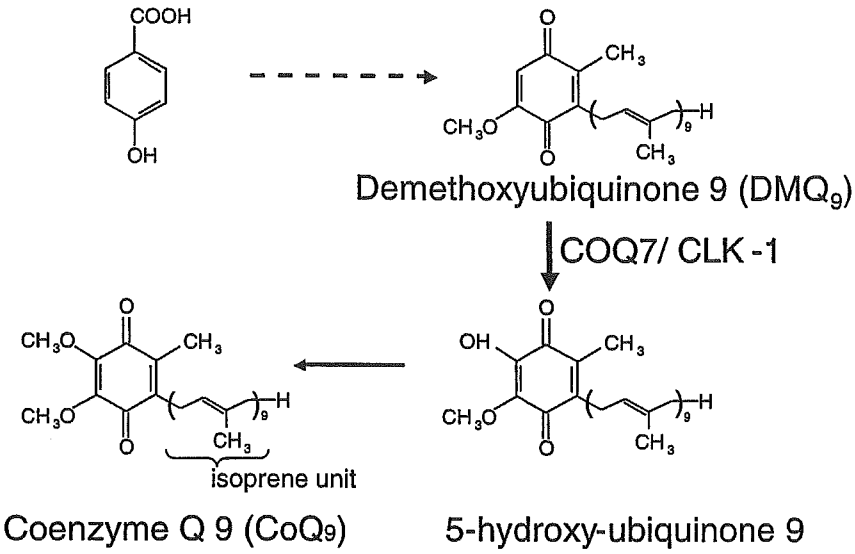

Fig. 1 Pathway for the biosynthesis of CoQ_9. The proposed pathway for CoQ_9 biosynthesis in eukaryotes is as previously described (Marbois & Clarke, 1996). COQ7/CLK-1 is essential for the step in which demethoxyubiquinone 9 (DMQ_9) is hydroxylated at the 5-position and converted to 5-hydroxy-ubiquinone.

Results

COQ7-transgene rescues the embryonic lethality of COQ7-deficient mice

To rescue the embryonic lethality of COQ7-deficient mice, we generated prion promoter-driven COQ7/CLK-1 transgenic mice (Fig. 2A). This prion promoter is capable of driving low levels of transgene expression in various tissues, with the highest expression occurring in the brain and heart (Borchelt *et al.*, 1996). We therefore assumed that the brain and heart were essential to the rescue from embryonic lethality.

To generate Tg mice with a *coq7–/–* background, we crossed *coq7+/–* mice with COQ7 Tg mice. Subsequently, *coq7+/–* mice expressing the COQ7 transgene were crossed with *coq7+/–* mice (Fig. 2B). COQ7-deficient mice were successfully rescued from embryonic lethality by intercrossing with two independent transgene lines, Tg23 and Tg96. COQ7-deficient mice were born following Mendelian rules and grew without obvious abnormalities; there were no differences in body weight at the age of 8 weeks among *coq7+/+* mice with COQ7 Tg, *coq7–/–* mice with COQ7 Tg and wild-type mice (data not shown). Western blot analysis indicated that Tg23 exhibited a lower COQ7 expression and Tg96 exhibited a higher COQ7 expression in various tissues (Fig. 2C). Immunohistochemical studies also indicated that transgenic COQ7 expression in the kidneys of Tg96 mice was comparable with that of endogenous COQ7 in the kidneys of wild-type mice. Although COQ7 Tg expression in the kidney is controlled by the prion promoter, the localization of transgenic COQ7 is indistinguishable from that of endogenous COQ7 (data not shown).

Rescued mice showed decreased CoQ_9

To investigate whether COQ7 transgene expression rescues CoQ_9 deficiency in COQ7-deficient mice, we measured the amount of CoQ_9, CoQ_{10} and DMQ_9 (hereafter called quinones) in various tissue homogenates of Tg23 and Tg96 mice using HPLC. In reverse-phase HPLC of quinones from kidney mitochondria, extracts from Tg23 and Tg96 mice, as well as wild-type mice, yielded a major peak at 16.1 min and a minor peak at 23.2 min, corresponding to CoQ_9 and CoQ_{10}, respectively (Fig. 3).

Table 1 Quantities of quinones in the kidneys and liver of Tg96 and Tg23 mice

	n	CoQ_9	DMQ_9	CoQ_{10}
Kidney				
WT	4	3.04 ± 0.24	n.d.	0.51 ± 0.13
Tg96	4	1.81 ± 0.44*	0.60 ± 0.23	0.41 ± 0.11
WT	4	2.72 ± 0.62	n.d.	0.46 ± 0.14
Tg23	3	2.10 ± 0.34	0.36 ± 0.20	0.30 ± 0.11
Liver				
WT	4	0.59 ± 0.14	n.d.	0.015 ± 0.004
Tg96	4	0.52 ± 0.05	n.d.	0.014 ± 0.002
WT	4	0.44 ± 0.13	n.d	0.015 ± 0.009
Tg23	4	0.42 ± 0.16	0.021 ± 0.016	0.013 ± 0.006

Values indicate nmol (mg protein)$^{-1}$. The standard deviation represents differences between analytical mice.
*$P < 0.05$ when compared with wild-type mice; n.d., not determined.

In total homogenates from the kidneys of Tg96 mice, the amount of CoQ_9 (1.81 ± 0.44 nmol mg^{-1} protein) was significantly depressed by 60% compared with wild-type mice (3.04 ± 0.24 nmol mg^{-1} protein), and the amount of CoQ_{10} was slightly depressed (0.41 ± 0.11 vs. 0.51 ± 0.13 nmol mg^{-1} protein) (Table 1). Although the precise validation of the quantified CoQ_9 would need the internal standard in the extraction process as described previously (Jonassen *et al.*, 2001), the standard deviation observed among samples from wild-type animals (Table 1) suggested negligible variations in the recovery of CoQ_9 in this assay. In the kidneys of Tg23, the amounts of CoQ_9 or CoQ_{10} in the mutant mice were not significantly different as

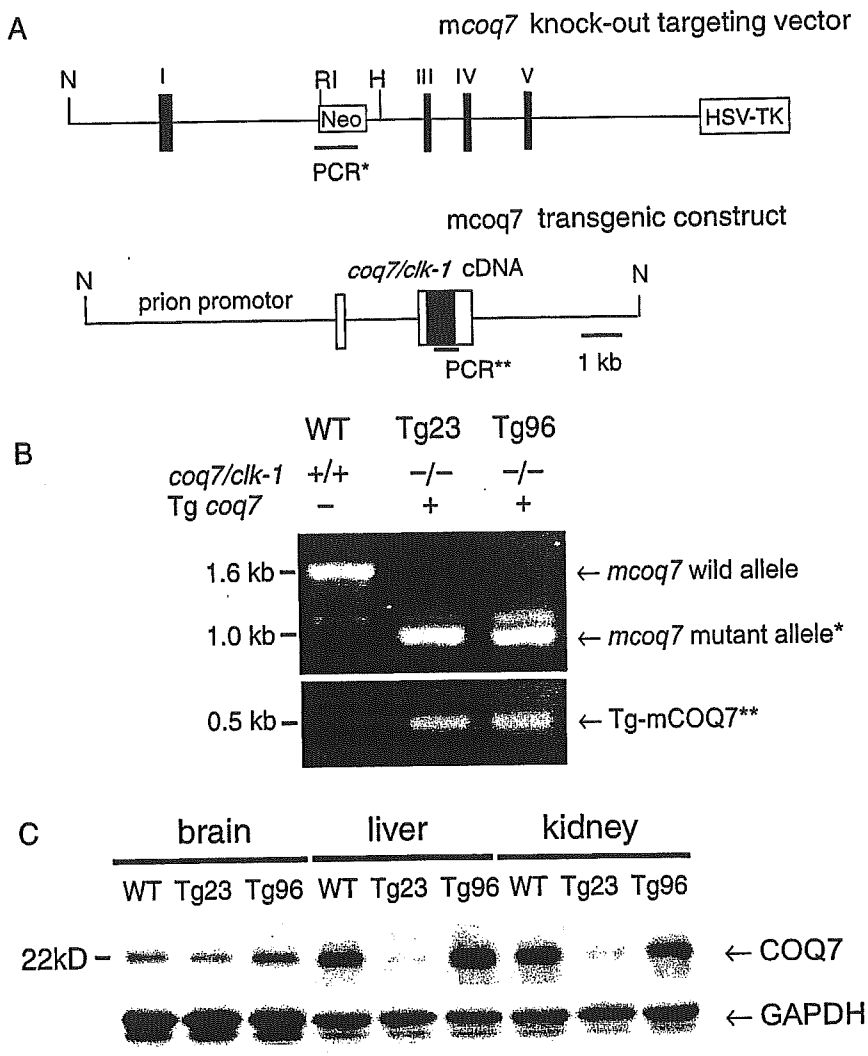

Fig. 2 Generation of COQ7 transgenic mice. (A) Schematic presentation of the mouse *coq7* knock-out targeting vector and mouse *coq7* transgenic construct. In the targeting vector, exons from the *coq7* genome are indicated by closed boxes, and the open boxes indicate the neomycin resistance cassette (Neo) and HSV-TK gene. The bar (*) indicates the fragment of *coq7* mutant allele used for PCR amplification. In the transgenic construct, mouse *coq7* cDNA was cloned into the mouse prion promoter construct (Borchelt *et al.*, 1996). Closed boxes represent mouse COQ7 cDNA, and open boxes are exon sequences of the prion. The bar (**) indicates the fragment used for PCR amplification. N; *Not*I. (B) Genotypic analysis of COQ7-deficient mice with the COQ7 transgene. The genotypes of *coq7/clk-1* were confirmed by PCR (upper panel) (Nakai *et al.*, 2001). The COQ7 transgene was confirmed by PCR with the COQ7-transgene-specific primers (lower panel) as described in the Experimental procedures. (C) Western blot analysis of COQ7-deficient mice with the COQ7 transgene. Tissue extracts (40 μg) were separated on 15% polyacrylamide-gel and COQ7 proteins were analysed by anti-mouse *coq7/clk-1* antibody. The 22-kDa molecule is recognized by anti-mouse *coq7/clk-1* antibody. Rescued COQ7-deficient mice showed detectable COQ7 expression in the brain, liver and kidneys.

Table 2 Quantities of quinones in the kidneys and liver mitochondria of Tg96 and Tg23 mice

	n	CoQ_9	DMQ_9	CoQ_{10}
Kidney				
WT	5	4.39 ± 0.80	n.d.	0.80 ± 0.10
Tg96	5	2.29 ± 0.79*	1.16 ± 0.49	0.55 ± 0.25
WT	3	4.77 ± 0.19	n.d.	0.88 ± 0.17
Tg23	3	4.65 ± 1.01	1.24 ± 0.18	0.93 ± 0.21
Liver				
WT	10	1.24 ± 0.19	n.d.	0.04 ± 0.01
Tg96	10	0.95 ± 0.37**	n.d.	0.03 ± 0.01

Values indicate nmol (mg mitochondrial protein)$^{-1}$. The standard deviation represents differences between analytical mice.
*$P < 0.005$, **$P < 0.05$; n.d., not determined

compared with wild-type mice (Table 1). In the liver of Tg96 and Tg23 mice, the amounts of CoQ_9 or CoQ_{10} were not significantly different as compared with wild-type mice (Table 1).

We next measured the amounts of quinones in the kidney and liver mitochondria of Tg23 and Tg96 mice (Table 2). In mitochondria from the kidney of Tg96 mice, the amount of CoQ_9 (2.29 ± 0.79 nmol mg^{-1} protein) was significantly depressed by 52% compared with the wild-type (4.39 ± 0.80 nmol mg^{-1} protein), and the amount of CoQ_{10} was slightly depressed (0.55 ± 0.25 vs. 0.80 ± 0.10 nmol mg^{-1} protein) (Table 2). In the mitochondria of kidney from Tg23 mice, the amount of CoQ_9 or CoQ_{10} was not significantly different (Table 2). In the mitochondria of liver from Tg96 mice, the amount of CoQ_9 was significantly depressed (0.95 ± 0.37 vs. 1.25 ± 0.19 nmol mg^{-1} protein), but the amount of CoQ_{10} was not significantly different (Table 2). These data indicate that the transgenic expression of COQ7 rescues CoQ_9 deficiency in mitochondria, but only partly rescues that found in the kidney and liver of Tg96 mice.

Accumulation of DMQ_9 in rescued COQ-deficient mice

We previously reported that COQ7-deficient mice fail to synthesize CoQ_9, but instead yield DMQ_9 (Nakai *et al.*, 2001). In HPLC, the mitochondrial fraction from the kidneys of Tg23 and Tg96 mice yielded additional peaks at 14.7 min (Fig. 3, Table 2). Mass spectrometric analysis of these peaks revealed that they

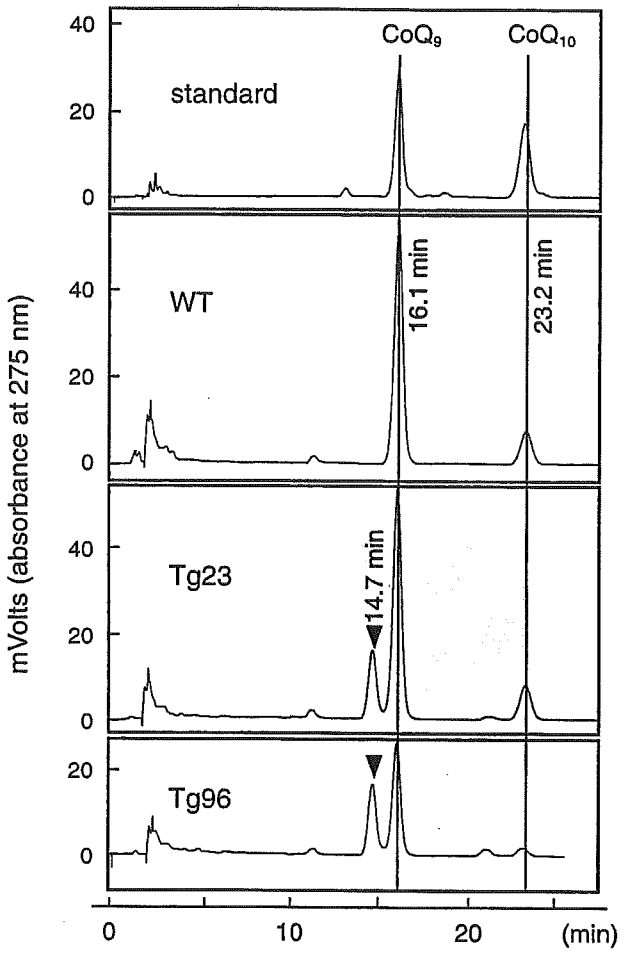

Fig. 3 Reverse-phase HPLC of CoQ from kidney mitochondria of Tg23, Tg96 and wild-type mice. The standards, CoQ_9 and CoQ_{10}, are shown in the upper panel. The two peaks, from Tg23 or Tg96 mice, appeared 1.4 min earlier than that for CoQ_9. Using mass spectrometric analysis, this additional peak at 14.7 min (arrowhead) was found to be identical to DMQ_9 (Nakai *et al.*, 2001). The CoQ_9 peak area in Tg96 is less than that of wild-type and Tg23 mice. One hundred micrograms of mitochondrial protein was used in each experiment.

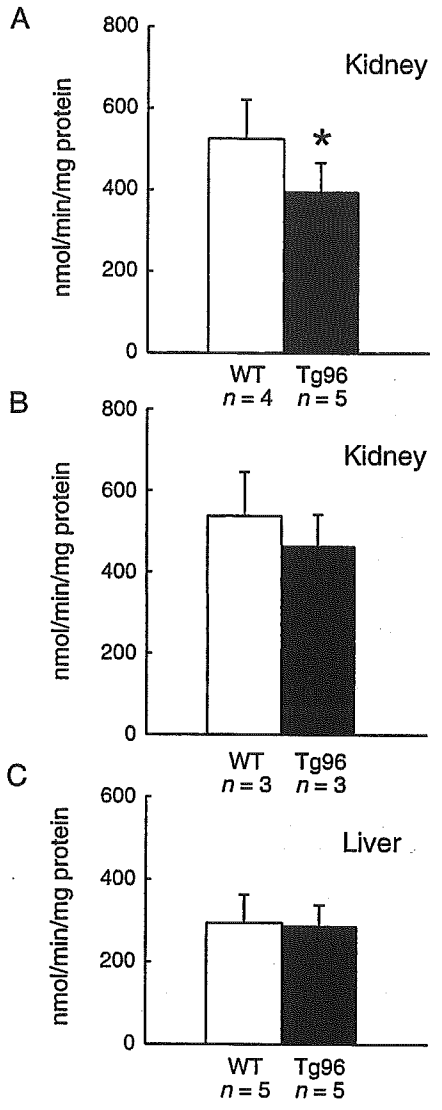

Fig. 4 Enzymatic activities of mitochondria in rescued mice. (A) Activities of respiratory chain enzymes in the kidneys of Tg96 mice. Enzyme activities are shown as nmol of the electron acceptor (cytochrome *c*) min^{-1} mg^{-1} mitochondrial protein. Succinate-cytochrome *c* reductase activities in the kidneys of Tg96 mice decreased significantly (*$P < 0.05$). Error bars represent the standard deviation. (B) Activities of succinate-cytochrome *c* reductase in the kidneys of Tg23 mice. There was no significant change in enzyme activities. (C) Activities of succinate-cytochrome *c* reductase in the liver of Tg96 mice. There was no significant change in enzyme activities.

represent DMQ_9 (Nakai *et al.*, 2001). Liver homogenates of Tg23 also demonstrated this peak, although that of Tg96 did not (Table 1). In mitochondria from Tg96 and Tg23 kidneys, a large amount of DMQ_9 was present, whereas in mitochondria from the liver of Tg96 DMQ_9 was not found (Table 2). These results indicate that DMQ_9 accumulated in mitochondria of rescued COQ7-deficient mice.

Rescued mice with a depressed CoQ_9 content show lower mitochondrial respiratory activities

To investigate whether transgene expression of COQ7 rescues mitochondrial functions in COQ7-deficient mice, mitochondrial enzyme activities requiring CoQ as an electron transporter (CoQ-responsive respiratory enzyme) were assayed in Tg23 and Tg96 mice. The enzyme is succinate-cytochrome *c* reductase (complex II + III), which uses succinate as a substrate. Succinate-cytochrome *c* reductase activity in the kidneys of Tg96 mice was significantly depressed compared with that of wild-type mice (533.9 ± 82.0 vs. 396.8 ± 62.3 nmol min^{-1} mg^{-1} protein) (Fig. 4A). Mitochondria from the kidneys of Tg23 mice showed no changes in enzyme activity (Fig. 4*B*). Mitochondria from the liver of Tg96 mice also showed no changes in enzyme activity (Fig. 4C). These results suggest that kidney mitochondria with a lower CoQ_9 content exhibit reduced succinate-cytochrome *c* reductase activity.

To understand further the relationship between quinones and respiratory chain enzyme activity, we evaluated the correlation

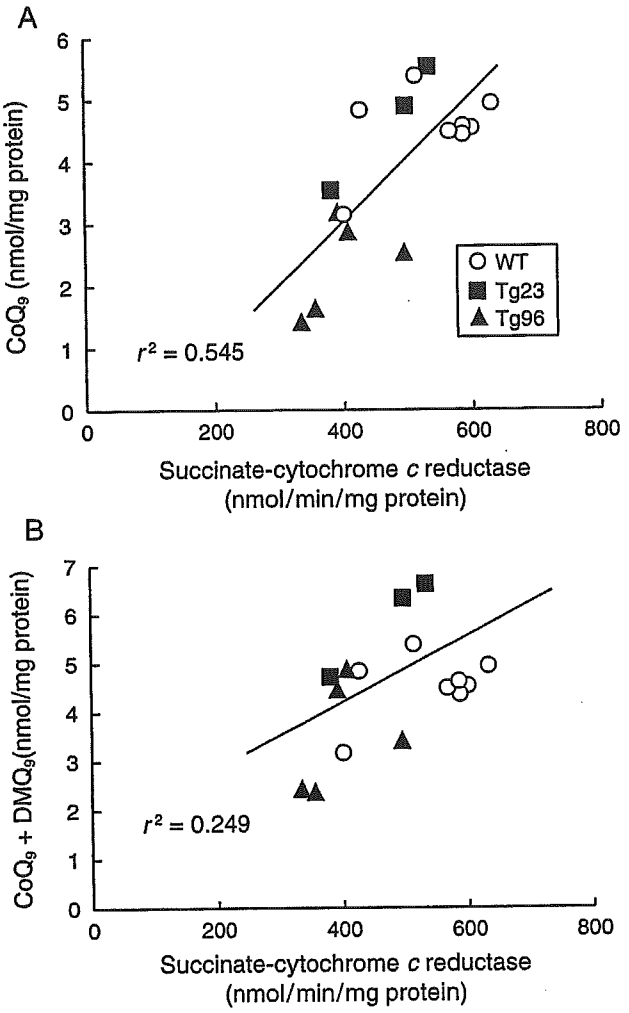

Fig. 5 Correlations between the amounts of quinones and the activities of succinate-cytochrome c redutase in kidney mitochondria in Tg23 and Tg96 mice. (A) A good correlation exists between the activity of succinate-cytochrome c redutase and CoQ_9 content (Pearson's correlation coefficient: $r^2 = 0.545$) (B) However, the amount of $CoQ_9 + DMQ_9$ is weakly correlated with the activity of succinate-cytochrome c redutase ($r^2 = 0.249$).

between succinate-cytochrome c reductase activity and the amount of quinones in kidney mitochondria. Over the physiological range of CoQ_9 content of kidney mitochondria, succinate-cytochrome c reductase activity rises in proportion to CoQ_9 content (Pearson's correlation coefficient; $r^2 = 0.545$, Fig. 5A). However, the amount of $CoQ_9 + DMQ_9$ is weakly correlated with succinate-cytochrome c reductase activity ($r^2 = 0.249$, Fig. 5B). These results indicate that succinate-cytochrome c reductase activity depends on CoQ_9 content in mitochondria of the kidney, whereas DMQ_9 is less likely to contribute to the enzyme activity.

Rescued mice with lower CoQ_9 content generate lower levels of ROS

Because the activities of mitochondrial respiratory enzymes closely correlate with ROS production, we measured the generation of ROS such as $O_2^{\bullet-}$ and H_2O_2 as a deleterious factor of lifespan using the chemiluminescent probes MPEC and DCFH-DA. MPEC and DCFH-DA are primarily sensitive toward $O_2^{\bullet-}$ and H_2O_2, respectively. Kidney mitochondria from Tg96 mice generated less $O_2^{\bullet-}$ (88% of that of wild-type mice) and H_2O_2 (59% of that of wild-type mice) as indicated by the white bars ($P < 0.05$) in Fig. 6(A,B). ROS generation increased in mitochondria when the complex II substrate succinate was added, as previously reported (Liu *et al.*, 2002). When succinate was added, $O_2^{\bullet-}$ (82%) and H_2O_2 (55%) generation from the kidneys of Tg96 was lower than that of wild-type mice (Fig. 6A,B, black bars, $P < 0.05$). These results indicate that mitochondria with a depressed CoQ_9 content generate less ROS.

Discussion

In this study, we successfully rescued COQ7-deficient mice from embryonic lethality by transgenic expression of COQ7 under control of the prion promoter (Fig. 2C). We established two independent rescued lines, Tg23 and Tg96. We detected a comparable level of CoQ_9 in the kidney of Tg23 with that of wild-type mice, whereas Tg96 mice showed less CoQ_9 compared with wild-type mice. In addition, we also detected the definitive DMQ peak, which was identical to that detected in COQ7-deficient mice and *clk-1* in *C. elegans*.

There is an unexplained variance between the expression levels of the COQ7 polypeptide (Fig. 2C) and the level of CoQ_9 (Tables 1 and 2), or the activity of succinate-cytochrome c reductase (Fig. 4) detected in the kidneys from transgenic lines Tg23 and Tg96. That is, Tg96 with the highest gene expression of *clk-1* appears to have the lowest levels of CoQ_9 and the lowest levels of succinate-cytochrome c reductase activity. One possible explanation is that the enhanced expression of COQ7 polypeptide in the kidney of Tg96 may only come from specific renal tubular cells that would not contribute to the overall enzyme activity of succinate-cytochrome c reductase in the kidney. Another possibility is that the protein expression of COQ7 does not necessarily correlate with the enzyme activity in the case of exogenously expressed COQ7 in transgenic mice.

CoQ is an electron transporter between complex I and complex III, or between complex II and complex III. To understand the influence of depressed CoQ on mitochondrial functions, we measured the activities of respiratory chain enzymes in Tg23 and Tg96 mice. We observed the depressed enzyme activities of CoQ-responsive respiratory chain in the isolated mitochondria from the kidney of Tg96 (Fig. 4A), indicating that succinate-cytochrome c reductase activity significantly correlates with CoQ_9 contained in the mitochondria (Fig. 5). Human cases with CoQ_{10} deficiency have been reported, and these patients usually develop symptoms of mitochondiral encephalomyopathy from early childhood (Ogasahara *et al.*, 1989; Boitier *et al.*, 1998; Sobreira *et al.*, 1997; Di Giovanni *et al.*, 2001). They showed depressed levels of CoQ_{10} in muscle, ranging from 4% to 39% of healthy controls, as well as depressed NADH– and succinate-cytochrome c reductase activities. Interestingly,

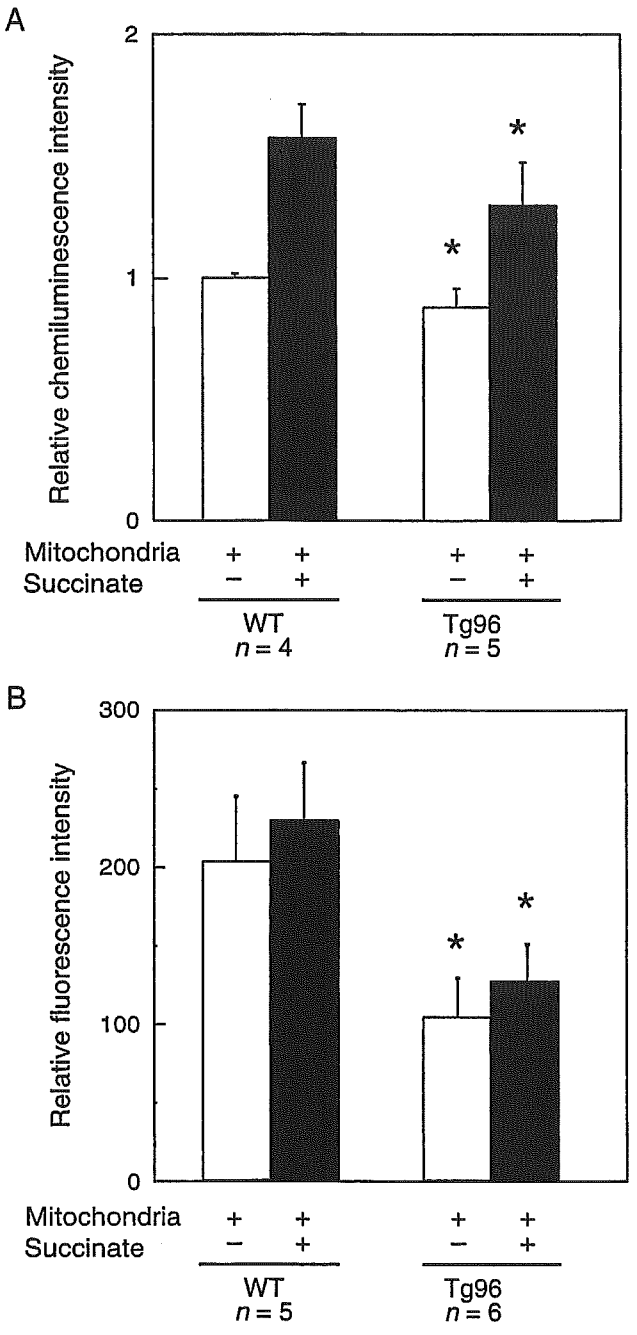

Fig. 6 Reduced generation of reactive oxygen species (ROS) in kidney mitochondria of rescued COQ7-deficient mice. (A) Mitochondrial $O_2^{\bullet-}$ generation in the kidneys of Tg96 mice. Using the chemiluminescent probe MPEC, $O_2^{\bullet-}$ generation from wild-type and Tg96 mice was measured in the presence or absence of 1.5 mM succinate. Error bars represent the standard deviation. On each experimental day, each value was calculated on the basis of the average of the chemiluminescence intensity of wild-type mice in the presence of 100 μg of mitochondria only (white bar). With mitochondria only (white bars, $*P < 0.05$) or with succinate as a substrate (black bars, $*P < 0.05$), kidney mitochondria from Tg96 showed lower generation than that of wild-type mice. (B) Mitochondrial H_2O_2 generation was also measured using 2′,7′-dichlorofluorescin diacetate (DCFH-DA) as a fluorometric probe. The emitted fluorescence from this probe is directly proportional to the concentration of H_2O_2 (Wang & Joseph, 1999). The relative fluorescence intensity from wild-type and Tg96 mice was measured in the presence or absence of 2 mM succinate. Error bars represent the standard deviation. For all experimental conditions, kidney mitochondria from Tg96 showed lower H_2O_2 generation than that of wild-type mice ($*P < 0.05$).

succinate-cytochrome *c* reductase activity was also slightly depressed in *clk-1* mutants (Felkai *et al.*, 1999; Miyadera *et al.*, 2001), whereas it was severely depressed in embryonic stem (ES) cells derived from embryos of COQ7-deficient mice (Levavasseur *et al.*, 2001). The *clk-1* mutant worms absorb CoQ_8 from dietary *E. coli* (Jonassen *et al.*, 2001) while endogenously generating DMQ_9 in its mitochondria (Miyadera *et al.*, 2001). COQ7-deficient ES cells, however, only represent DMQ_9 in their mitochondria (Levavasseur *et al.*, 2001). Taken together, our results suggest that DMQ_9 has only a minor physiological role as an electron transporter in mitochondria. In support of this idea, a DMQ derivative shows a depressed potential for transporting electrons compared with the CoQ derivative *in vitro* (Gu *et al.*, 1990). Alternatively, DMQ_9 may possibly compete with CoQ_9 in the mitochondrial respiratory chain if DMQ_9 and CoQ_9 coexist in the same mitochondria. In this case, DMQ has an inhibitory effect on the electron transfer in oxidative phosphorylation as well as on the generation of ROS.

The domains of NADH- and succinate-cytochrome *c* reductase are suggested as the main sites of $O_2^{\bullet-}$ generation (Chance *et al.*, 1979; Turrens *et al.*, 1985). In addition, the ubiquinone binding site in complex III and the flavin mononucleotide group binding site in complex I have been also reported to be ROS-generating sites (Turrens & Boveris, 1980; Turrens *et al.*, 1985). It is thus noteworthy that the rate of $O_2^{\bullet-}$ generation in cardiac submitochondrial particles is directly related to the relative amount of CoQ_9 (Lass *et al.*, 1997). These reports are therefore consistent with data demonstrating that Tg96 mice showed a depressed rate of $O_2^{\bullet-}$ generation from kidneys (Fig. 6), which concomitantly showed the depressed enzyme activities of CoQ-responsive respiratory chain (Figs 4 and 5). In addition, Tg96 mice generated significantly lower amounts of H_2O_2 than wild-type mice, and they also generated slightly lower amounts of $O_2^{\bullet-}$ (Fig. 6). Because $O_2^{\bullet-}$ generated in the mitochondria is rapidly converted to H_2O_2 by endogenous manganese superoxide dismutase (Mn-SOD), it is difficult to detect *de novo* $O_2^{\bullet-}$ accurately (Forman & Azzi, 1997). It is reported that SOD activity in the *clk-1* mutant is lower than that of the wild-type (Braeckman *et al.*, 2002). We assumed that lower amounts of CoQ_9 in Tg96 mice limit the rate of electron flow and regulate respiratory chain enzyme activities at the CoQ site in the mitochondrial respiratory chain. We consider that retarded electron flow influences the rate of electron leakage and $O_2^{\bullet-}$ generation.

The mechanism by which *clk-1* extends lifespan is still to be defined. Recently, RNA interference (RNAi) on the genes for the respiratory complex (Dillin *et al.*, 2002) as well as the genes involved in CoQ synthesis (Asencio *et al.*, 2003) such as *clk-1* extend lifespan in *C. elegans*. These data indicate that the extension of animal lifespan might be attributed to the depressed activities of mitochondrial enzymes (Dillin *et al.*, 2002), or alternatively to the suppressed generation of superoxide caused by depressed CoQ (Asencio *et al.*, 2003). Contrary to the latter hypothesis, Braeckman *et al.* (2002) reported that the generation of ROS in *clk-1* (*e2519*) was slightly elevated when measured using lucigenin luminescence.

In this study, we established a novel mammalian model system to investigate the relationship between *coq7/clk-1* and mitochondrial functions. Our COQ7-deficient model mice with a COQ7 transgene represent both DMQ and CoQ in mitochondria. The lower amounts of CoQ_9 in this model may cause a decrease in the enzyme activities of the mitochondrial respiratory chain as well as the decrease in ROS generation. Thus, an animal model carrying variable amounts of quinones in mitochondria would be useful for the analysis on the mechanism of lifespan that is controlled by mitochondrial functions.

Experimental procedures

Generation of COQ7 transgenic (Tg) mice

A COQ7/CLK-1 Tg construct was assembled by inserting mouse *coq7* cDNA into the *Xho*I site of the transgenic vector MoPrP.Xho (Borchelt *et al.*, 1996). After linearization with *Not*I, the Tg construct was isolated by gel electrophoresis and purified using a Gene Clean II Kit (Bio 101, Vista, CA, USA). Purified Tg construct was then microinjected into the pronuclei of BDF1 mice (Nihon SLC, Shizuoka, Japan). Tail DNA from potential founder mice was screened by PCR amplification with a sense primer (5′-GTGGGATCAAGAGAAGAACC-3′) and antisense primer (5′-AGAAGCAAGAATGAGAACCACCTC-3′). Four lines of germline mice transmitted Tg, Tg23, Tg82, Tg95 and Tg96, and were selected for further analysis. To generate COQ7 Tg mice with a *coq7*–/– background, we crossed *coq7*+/– mice with COQ7 Tg mice. In all four lines, the transgene was successfully transmitted to the germline of F1 mice. Subsequently, *coq7*+/– mice expressing the COQ7 transgene were crossed with *coq7*+/– mice to generate COQ7 Tg mice with a *coq7*–/– background. Finally, two lines of COQ7 Tg mice (Tg23 and Tg96) successfully rescued the embryonic lethality of COQ7-deficient mice.

Preparation of anti-mouse *coq7/clk-1* antibody

A 537-bp fragment containing mouse *coq7/clk-1* cDNA fragment (39–217 amino acid residues) starting from the second ATG was PCR-amplified with primers (*Not*I-anchored primer: 5′-ATAAGAATGCGGCCGCACCTTAGACAATATTAACCGGG-3′ and *Bam*HI-anchored primer: 5′-CGGGATCCAAACCTTTCTGA-TAAATA-3′) from the plasmid pcDNA3.1 (Invitrogen, CA, USA) containing full-length mouse *coq7/clk-1* cDNA (GeneBank Accession number AF098949) (Takahashi *et al.*, 2001). A *Not*I/*Bam*HI-restricted PCR product was inserted into a *Not*I/*Bam*HI-restricted pIVEX2.4a vector (Roche, Mannheim, Germany) for *in vitro* translation. Recombinant mouse *coq7/clk-1* (39–217) proteins were synthesized at 30 °C for 24 h at 120 r.p.m. stirring using a Rapid Translation System RTS500 *E. coli* HY kit (Roche). The N-terminal His-tagged proteins were precipitated by incubation and collected by centrifugation. The pellet was washed with phosphate-buffered saline (PBS) and dissolved into urea lysis buffer (8 M urea, 20 mM sodium phosphate (pH 7.8), and 500 mM NaCl). The urea-soluble fraction was purified by an Ni^{2+}-charged HiTrap chelating HP column (Amersham Pharmacia Biotech, Uppsala, Sweden). After dialysis against PBS, the purified protein was injected three times into a New Zealand white rabbit with Freund's complete or incomplete adjuvant. A week after the last injection, antiserum was collected and the IgG fraction was purified using a HiTrap protein G HP column (Amersham Pharmacia Biotech).

Western blotting of COQ7

Mouse tissues were homogenized in 10 mM sodium phosphate (pH 7.2), 1 mM EDTA, 0.2 mM pheneylmethylsulphonyl fluoride and 0.5 μg mL^{-1} leupeptin containing 1% Triton X-100. The homogenates were centrifuged at 20 000 ***g*** for 10 min at 4 °C and the supernatants were subjected to Western blotting. Procedures of Western blotting were as previously described (Takahashi *et al.*, 2001) except for the use of anti-mouse *coq7/clk-1* antibody.

Analysis of coenzyme Qs (quinones)

Quinones were extracted from tissue homogenates by ethanol/*n*-hexane (2 : 5, v/v) as previously described (Nakai *et al.*, 2001) and subjected to reverse-phase high-performance liquid chromatography (HPLC) (Devolosil C-30-UG-5 3.0 × 150 mm, Nomura Chemical, Aichi, Japan). Quinones were eluted by ethanol/methanol (1 : 1, v/v) at 0.43 mL min^{-1}. The amount of CoQ_9 or CoQ_{10} was determined by comparing them with CoQ_9 and CoQ_{10} standards (Sigma, St Louis, MO, USA), respectively. The amounts of DMQ_9 were also calculated by comparing them with CoQ_9 standards.

Mitochondrial isolation

Mitochondria were isolated from the supernatants by differential centrifugation (Lash & Sall, 1993). Mouse kidney or liver was homogenized in 10 volumes (w/v) of Buffer-1 (0.25 M sucrose, 0.2 mM EDTA buffer) using a Teflon pestle with a 0.3-mm clearance by nine up and down strokes at 1200 r.p.m. on a Digital homogenizer HOM (Asone, Osaka, Japan). All operations were performed at 4 °C. The homogenates were centrifuged at 100 ***g*** for 5 min. After a 0.5 volume of Buffer-2 (0.35 M sucrose, 0.2 mM EDTA) was added to each supernatant, the solutions were centrifuged at 800 ***g*** for 15 min. The supernatants were then centrifuged at 9000 ***g*** for 10 min and the pellets were resuspended in the same volume of Buffer-1. The suspensions were washed at 9000 ***g*** for 7 min and the mitochondrial fraction was resusupended at a final concentration of 10–15 mg mitochondrial protein/mL in Buffer-1. Protein concentration was determined using a DC protein Assay kit (Bio-Rad, CA, USA) according to the manufacturer's instructions.

Analysis of respiratory chain enzyme activities

Mitochondrial fractions of kidney and liver were stored at −30 °C for up to 1 month. Succinate-cytochrome *c* reductase activity (complex II + III) was measured as follows; the reduction of cytochrome *c* by complex III coupled to succinate oxidation through complex II was followed at 550–540 nm (extinction coefficient of 19.0 mM cm^{-1}) using a double-beam spectrophotometer, DU7500 (Beckman, CA, USA). In a 1-mL cuvette, a reaction mixture consisting of 40 mM potassium phosphate (pH 7.4), 20 mM succinate, 0.5 mM EDTA, 2 mM KCN and 100 μg kidney or liver mitochondrial protein was incubated at 30 °C for 20 min. The reaction was initiated by adding 30 μM cytochrome *c* and the reduction of cytochrome *c* was monitored as a linear absorbance increase for 3 min (Trounce *et al.*, 1996).

Determination of the generation of ROS in mitochondria

Superoxide anion generation was measured using the chemiluminescent probe MPEC (2-methyl-6-p-methoxyphenylethynylimidazopyrazinone) (ATTO, Tokyo, Japan). MPEC is a new imidazopyrazinone derivative that is sensitive and useful for measuring superoxide (Shimomura *et al.*, 1998). Mitochondrial proteins (100 μg) were added to 1 mL of assay buffer (50 mM HEPES (pH 7.4), 2 mM EDTA) containing 0.05 mM MPEC. The reaction mixture was placed in a Lumat LB9507 luminometer (Berthold Technologies, Bad Wildbad, Germany) and the chemiluminescence intensity of MPEC was measured for 1 min at room temperature. To measure superoxide anion generation from mitochondria with a respiratory substrate, 1.5 mM succinate as a substrate of respiratory complex II was added to the solution. The absolute chemiluminescence intensity used to quantify superoxide anion generation was calculated by subtracting the background intensity (in the absence of mitochondria). The experiments were repeated three times. Data analysis was performed as follows: the relative chemiluminescence intensity was calculated for each experimental day using the formula [chemiluminescence intensity for each condition/the average chemiluminescence intensity of the wild-type in the presence of 100 μg of mitochondria without substrate].

ROS including H_2O_2 were also measured by 2′,7′-dichlorofluorescin diacetate (DCFH-DA) (Molecular Probes, OR, USA), which was used as a fluorometric probe. When DCFH-DA crosses the mitochondrial membrane, it is cleaved to non-fluorescent DCFH by an esterase (Bejma & Ji, 1999). Furthermore, DCFH is oxidized to fluorescent dichlorofluorescin (DCF) by intramitochondrial ROS. The fluorescence emitted is directly proportional to the concentration of H_2O_2 (Wang & Joseph, 1999). In our experiment, mitochondrial proteins (50 μg) were added to 1 mL of assay buffer (130 mM KCl, 5 mM $MgCl_2$, 20 mM Na_2HPO_4, 20 mM Tris-HCl, 30 mM glucose (pH 7.4)) containing 5 μM DCFH-DA with or without 2 mM succinate. The reaction mixture was incubated at 37 °C for 15 min, and then centrifuged at 12 000 ***g*** for 8 min to discard supernatant containing DCFH-DA that did not cross the mitochondrial membrane. The mitochondrial pellets were resuspended in the assay buffer. DCF formation was quantified at an excitation wavelength of 488 nm and emission wavelength of 525 nm using a Spectra Max Gemini XS fluorescent microplate reader (Molecular Devices, CA, USA) at 37 °C. The experiments were repeated three times.

Statistical analysis

Data on CoQ content, mitochondrial respiratory chain enzyme activity and relative chemiluminescence intensity of MPEC were separately analysed using Student's *t*-test.

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Original article

Pathologic characteristics of gastric cancer in the elderly: a retrospective study of 994 surgical patients

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Abstract

Background. **The clinicopathologic features of gastric carcinoma in elderly people have been reported previously. The present study examined the patterns and distribution of gastric carcinomas in the elderly, especially in patients aged 85 and older.**

Methods. **A retrospective study of 994 consecutive Japanese patients aged 65 years or older was performed. In this group, a total of 1147 lesions were analyzed. Pathological findings in the very old group (older than 85 years; $n = 126$) were compared with those in younger groups (65–74 years [young-old group]; $n = 356$) and (75–84 years [middle-old group]; $n = 512$).**

Results. **While the male-to-female ratio significantly decreased with advancing age, the relative odds of gastric cancer in men were higher than those in women in all age groups. In the very old group, cancer of the lower third of the stomach tended to increase with advancing age, and accounted for 43.7% of cases. In the population overall, differentiated-type adenocarcinoma accounted for 89.6% in the early cancers and 50.3% in the advanced cancers. The proportion of cases involving differentiated-type carcinoma significantly increased with advancing age in early cancer and female advanced cancer cases, whereas no significant change was found in male advanced-cancer patients. In the very old group, lymph node metastasis was found in 5.4% of early cancers and 72.7% in advanced cancers. Multiple cancers significantly increased with advancing age ($P < 0.05$; 10.7% in the younger-old group, 12.7% in the middle-old group, and 19.0% in the very old group).**

Conclusion. **These results indicate that, in the very old group, gastric cancers showed a distal shift with predominantly differentiated-type carcinoma in the early stages and increased undifferentiated-type carcinomas in advanced stages. These results suggest increased histologic diversity with tumor growth. These findings have important implications for the screening and diagnosis of gastric cancer in the elderly.**

Key words Carcinoma · Stomach · Elderly · Differentiated-type carcinoma

Introduction

The incidence of gastric cancer in elderly people has recently been increasing in Japan due to the extension of the life span in the general population. This has been an ongoing trend despite an overall decrease in the incidence of gastric cancer in the country. It is estimated that gastric cancer in patients aged 65 years or older accounts for approximately 70% of total gastric cancers [1]. Moreover, age-adjusted death rates for stomach cancer have increased exponentially with aging [2]. This evidence indicates that the relationship of aging to cancer in elderly patients is of great importance.

The characteristic features of gastric carcinoma in elderly people have been reported by several investigators [3–9]. Previous studies have shown that gastric cancer involving the lower third of the stomach and histopathologically well-differentiated adenocarcinoma was significantly more prevalent in elderly patients [6,8, 10]. It is also reported that the frequency of advanced cancer in the elderly is higher than in young people [7], and multiple and metastatic cancers are more common in aged people [5,11]. However, only a few reports, using a relatively small number of cases, have examined the clinicopathologic features of gastric carcinoma in very old people. The present study focused on this point by examining the patterns and distribution of gastric carcinomas in the elderly, especially in patients aged 85 and older.

Patients and methods

A total of 994 consecutive patients aged 65 years or older, with a total of 1147 carcinomas of the stomach,

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were selected from patients at the Tokyo Metropolitan Geriatric Medical Center between January 1984 and December 2003. Of the 1147 carcinomas, 1135 lesions (982 patients) were surgically resected, and 12 lesions (12 patients; 11 intramucosal cancers and 1 cancer with submucosal invasion) were endoscopically resected. The tumors were histopathologically examined as part of the routine procedure. Resectability rates of gastric carcinoma in the hospital between May 1986 and December 1994 were 79.1% (155/196) in the young-old group (see below for age range), 75.7% (240/317) in the middle-old group, and 58.3% (56/96) in the very old group. In elderly patients who did not undergo resection of the tumor, some were found to have unresectable disease because of metastasis. These metastases included liver metastasis, peritoneal dissemination, and distant lymph node involvement. Other patients who did not undergo resection had severe complications, such as chronic heart failure, myocardial infarction, diabetes, and aspiration pneumonia.

We reviewed pathological protocols and microscopic slides in each case. We also analyzed records for age, sex, location of the tumor, histological type, depth of invasion, lymph node metastasis, and the presence of two or more gastric carcinomas. The anatomical site of gastric tumors was classified based on surgical records and pathological reports. Histological types were described on the basis of the *Japanese classification of gastric carcinoma* [12].

All patients were divided into three age groups: young-old group (65–74 years old; 356 patients with 413 lesions), middle-old group (75–84 years old; 512 patients with 583 lesions), and a very old group (85 years and older; 126 patients with 151 lesions). We compared the parameters in the very old group with those in the younger groups. Furthermore, we compared the data of the earlier decade (1984–1993) with those of the following decade (1994–2003) due to the long observation period.

Differences among groups were tested for statistical significance using a χ^2 test. A P value less than 0.05 (two-sided) was considered significant. Because the greater longevity of females may produce an apparent increase in the number of female patients with gastric cancer, the relative odds of gastric carcinoma in both sexes were calculated and compared with each other. The relative odds for males were calculated according to the following formula: $p\,(1 - p_0) / p_0\,(1 - p)$; p = ratio of male patients with gastric cancer in each age group, and p_0 = ratio of males in the general Japanese population in the same age group [13]. Female odds were calculated in the same way. The numbers of individuals in each age group in the general population during the period from 1985 to 2000 were obtained from the *Vital statistics of Japan* [1].

Results

The percentages of men decreased with age (66.1% in the young-old group, 53.5% in the middle-old group, and 50.8% in the very old group). The young-old group consisted of more men than women ($P = 0.0003$). The relative odds of having gastric cancers in men were higher than those in women in all age groups (Table 1). Site distribution of gastric cancers is shown in Table 1. Cancer of the lower third of the stomach in the very old group accounted for 43.7%. This was a slightly higher rate than in the younger groups. The percentage of cancer of the lower third of the stomach in women (41.5%) was higher than that in men (36.5%). Early-to-advanced cancer ratios were approximately equal in all age groups. Elevated-type cancer increased with age in both early and advanced cancers (Table 1). On the other hand, the percentage of type 4 cancer (diffuse-infiltrative type) in women (17.7%) was higher than that found in men (13.7%). In the very old group, differentiated-type adenocarcinoma accounted for 89.6% of the early cancers and 50.3% of the advanced cancers (Table 1). The proportion of differentiated-type carcinoma increased with advancing age in early cancer and female advanced cancers, whereas no significant change was found in male advanced cancer (Fig. 1). The proportion of differentiated-type carcinoma in men was

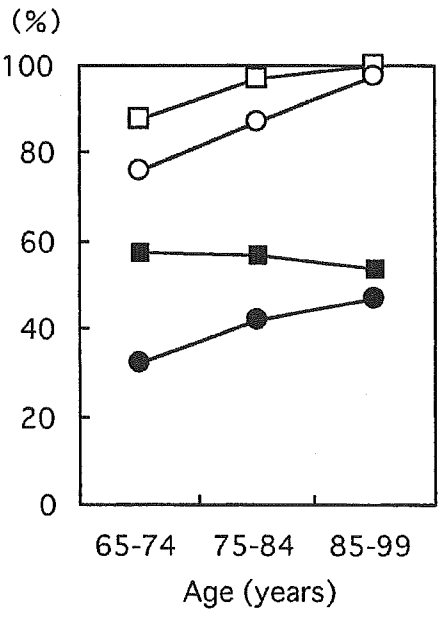

Fig. 1. Proportions of differentiated-type carcinoma in early and advanced cancers. Note increased proportion of differentiated-type carcinoma with advancing age in early and female advanced cancer, but there was no significant change in male advanced cancer. *Open squares*, male early cancer; *open circles*, female early cancer; *closed squares*, male advanced cancer; *closed circles*, female advanced cancer

Table 1. Characteristics of gastric cancer in 994 elderly patients

No. of lesions	Age groups, in years			
	65–74 (n = 413)	75–84 (n = 583)	85–99 (n = 151)	All (n = 1147)
Male:Female** (Percentages of men)	235:121 (66.1)	274:238 (53.5)	64:62 (50.8)	573:421 (57.7)
Relative odds of gastric cancer				
Male	2.41	1.89	112.09	1.90
Female	0.41	0.53	10.48	0.53
Tumor location				
Upper third	62 (15.0)	90 (15.4)	16 (10.6)	168 (14.6)
Middle third	172 (41.7)	258 (44.3)	61 (40.4)	491 (42.8)
Lower third	161 (39.0)	216 (37.0)	66 (43.7)	443 (38.6)
Whole	7 (1.7)	14 (2.4)	6 (4.0)	27 (2.4)
Remnant stomach	11 (2.7)	5 (0.9)	2 (1.3)	18 (1.6)
Early:Advanced cancers	203:210	272:311	74:77	549:598
Gross features				
Early cancer				
I	22 (10.8)	36 (13.2)	12 (16.2)	70 (12.8)
IIa	56 (27.6)	92 (33.8)	24 (32.4)	172 (31.3)
IIb	19 (9.4)	31 (11.4)	6 (8.1)	56 (10.2)
IIc	105 (51.7)	111 (40.8)	32 (43.3)	248 (45.2)
III	1 (0.5)	2 (0.8)	0	3 (0.6)
Advanced cancer				
1	11 (5.2)	21 (6.8)	7 (9.1)	39 (6.5)
2	71 (33.8)	109 (35.0)	25 (32.4)	205 (34.3)
3	68 (32.4)	99 (31.8)	24 (31.2)	191 (31.9)
4	35 (16.7)	42 (13.5)	14 (18.2)	91 (15.2)
5	25 (11.9)	40 (12.9)	7 (9.1)	72 (12.1)
Cancer ≥ 5 cm	187 (45.3)	291 (49.9)	72 (47.7)	550 (48.0)
Differentiated-type carcinoma				
Early cancer**	169/203 (83.3)	250/272 (91.9)	73/74 (98.6)	492/549 (89.6)
Advanced cancer	106/210 (50.5)	156/311 (50.2)	39/77 (50.6)	301/598 (50.3)
Lymph node metastasis				
Early cancer	14/203 (6.9)	19/272 (7.0)	4/74 (5.4)	37/549 (6.7)
Advanced cancer	153/210 (72.9)	237/311 (76.2)	56/77 (72.7)	446/598 (74.6)
Two or more cancers in the stomach*	38/356 (10.7)	65/512 (12.7)	24/126 (19.0)	127/994 (12.8)

* $P < 0.05$; ** $P < 0.01$
Values are numbers, with percentages in parentheses

higher than that in women in all age groups (Fig. 1). In the very old group, the proportion showing lymph node metastasis was 5.4% in early cancer and 72.7% in advanced cancers (Table 1). In early cancer, lymph node metastasis was more common in men than in women in all age groups (Fig. 2). In women, the rate of lymph node metastasis decreased with advancing age, whereas in men metastasis peaked in the middle-old group (Fig. 2). Multiple gastric cancers increased with advancing age ($P = 0.037$; 10.7% in the young-old group, 12.7% in the middle-old group, and 19.0% in the very old group). Multiplicity of gastric cancers in men was more frequent than in women (Fig. 3).

The proportion of lower-third gastric cancer in the most recent decade was higher than that in the previous decade (Fig. 4), even though age and sex distribution was not different between the two periods. Other than a decreasing trend in carcinoma of the upper third of stomach in the very old group, there were no significant changes between the two periods. Gross features, histology, and the early-to-advanced cancer ratio showed no significant difference between the two time periods. In the middle-old and very old groups, multiplicity of gastric cancer in the recent decade (1994–2003) was less common than in the previous decade (1984–1993), whereas in the young-old group there was a slight increase in the multiplicity of gastric cancer (Fig. 5) in the most recent decade.

Discussion

In the present study, we analyzed gastric cancers in elderly patients, from the standpoint of aging. We found that, in the very old group, tumors occurred predominantly in the lower third of the stomach; differentiated-

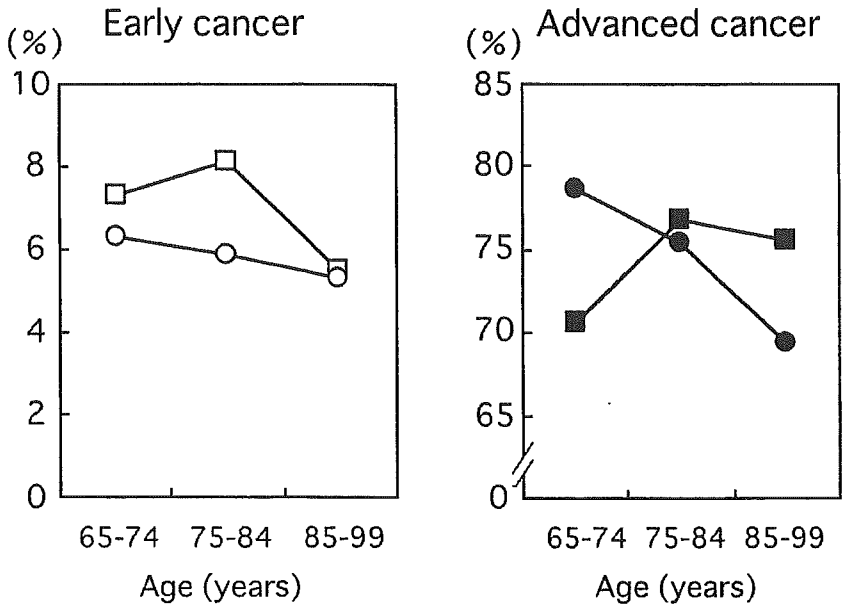

Fig. 2. The rates of lymph node metastasis in gastric cancer. In women, lymph node metastasis decreased with age. In men, the middle-old group (age 75–84 years) showed the most frequent rate of lymph node metastasis. *Open squares*, male early cancer; *open circles*, female early cancer; *closed squares*, male advanced cancer; *closed circles*, female advanced cancer

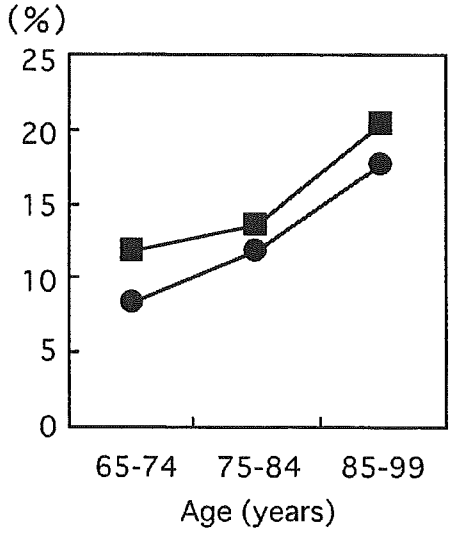

Fig. 3. Multiplicity of gastric cancers. Multiple gastric cancers increased with advancing age in both sexes. *Closed squares*, male; *closed circles*, female

type carcinoma was more common in early cancer; and multiple cancers increased with advancing age. Moreover, we found sex differences in histological type, lymph node metastasis, multiple cancers, and time trends in tumor location.

It is generally documented that gastric cancer in elderly patients is predominantly localized to the lower third of the stomach [6–9,14–18]. Previous reports demonstrated that the proportion (range, 42%–63%) of carcinomas in the lower third of the stomach in the elderly was higher than the proportion (range, 31%–44%) in the young [3,6–9,14–19]. Our data confirmed that, in patients aged 85 years or older, cancers were situated more often in the lower third of the stomach compared with the younger age groups. Thus, it is conceivable that the risk of developing carcinoma in the lower third of the stomach increases with advancing age. This trend held true even in individuals 85 years or older. On the other hand, there was no clear trend of risk patterns involving carcinoma of the upper third of the stomach. Some investigators have reported that gastric cancer involving the upper third of the stomach in the elderly was more common than in younger groups [6,7,15–17,19], whereas others found no significant difference or lower prevalence in the elderly group [3,8,14,18].

Several reports have indicated that differentiated-type or intestinal-type carcinomas are more common in older patients than in younger patients [3,5–8,14–16,18,19]. Our study demonstrated that this tendency was clear, especially in early-stage cancer. We also found that the proportion of differentiated-type carcinoma increased with advancing age, reaching a level greater than 95%. These findings confirm evidence presented in our previous report [8]. Our findings demonstrated that, in cases of advanced gastric cancer, the proportion of differentiated-type carcinomas was almost equal to that of undifferentiated-type carcinoma. These results suggest that gastric cancer in the elderly demonstrates increased histological diversity during growth [8].

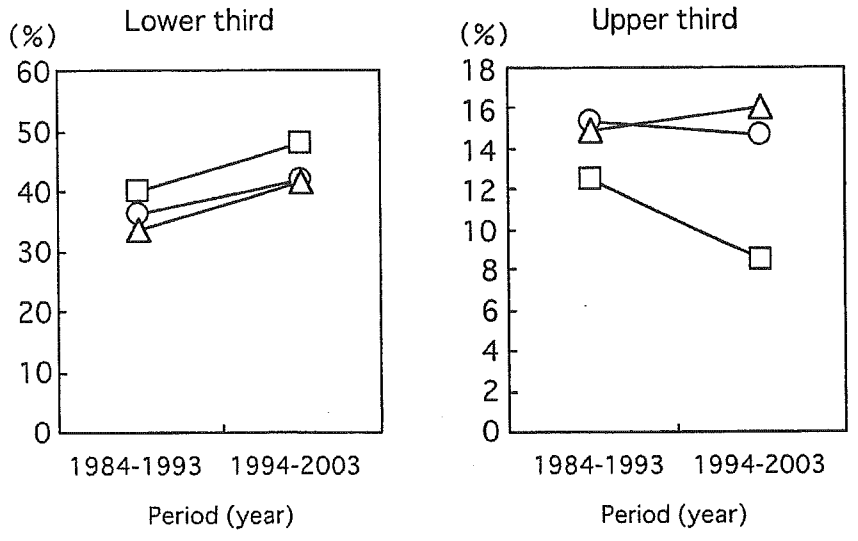

Fig. 4. Time trends in the proportion of upper- and lower-third gastric cancers over 20 years. Note the increasing trend in carcinoma of the lower third of the stomach and the decreasing trend in carcinoma of the upper third of the stomach in the very old group. *Open circles*, young-old group (65–74 years); *open triangles*, middle-old group (75–84 years); *open squares*, very old group (85–99 years)

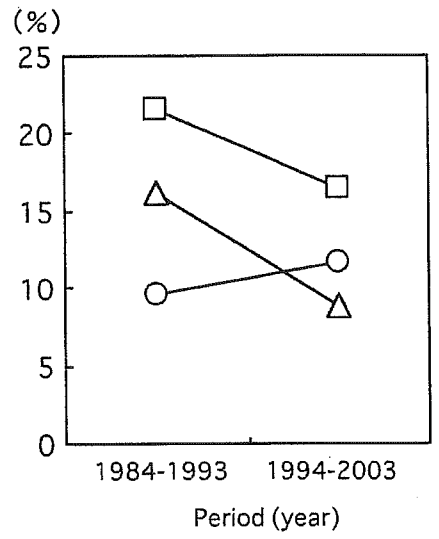

Fig. 5. Time trends in the proportion of multiple gastric cancers over 20 years. Note slight increasing trend in the young-old group and marked decreasing trend in the older groups. *Open circles*, young-old group (65–74 years); *open triangles*, middle-old group (75–84 years); *open squares*, very old group (85–99 years)

Previous reports have shown no significant difference in the incidence of lymph node metastasis between young and elderly patients with gastric cancer [3,5–7,14–19]. Most of these reports did not take the clinical or pathological stage of the disease into account, and the results could not be simply compared with each other. However, our findings, and a few other reports [6,14], suggest that the incidence of lymph node metastasis in the elderly is lower than that in younger patients in cases of early gastric cancer. Moreover, examination of autopsy cases of fatal gastric cancer showed that younger patients had a higher incidence of lymph node metastasis [20]. Thus, in the very old group, cancer tissue may metastasize less often to the regional lymph node compared with younger patients.

In general, multiple cancers are more common problems in elderly patients compared to younger populations [2]. The present study provided evidence that approximately 13% of our total population of elderly patients with gastric cancer had two or more cancers within the stomach, which is consistent with previous reports [5,11,15,21]. Although the incidence of multiple colorectal cancers was not significantly different among the age groups [13,22,23], multiple gastric cancers increased with advancing age [15,21,24].

Sex differences were recognized in site distribution, histologic type, and multiplicity. Our findings demonstrated a higher incidence of gastric cancer affecting the lower third of the stomach or whole stomach in females. Both differentiated-type and multiple gastric cancer sites were more common in men than in women in all age groups. In other studies, undifferentiated-type carcinoma was found more frequently in young women, whereas differentiated-type carcinoma primarily affected elderly men. Generally, undifferentiated-type carcinoma involves the whole stomach, which results in "linitis plasitca". Most multiple gastric cancers are composed of differentiated-type carcinoma [11]. Thus, sex differences in histologic type may play an important role in tumor location and multiplicity. Sex differences in histologic type may be due to intrinsic and environmental factors, such as sex hormones, bile acid, fatty acid, *H. pylori* infection, and alcohol intake. However, this issue remains unclear.

The anatomical site distribution of gastric cancer differed in time trends between the very old group (85 years or older) and the younger groups (65–84 years