with carnitine significantly inhibited the increase in those indicators.

Effects of carnitine on mitochondrial function and mtDNA in noncancerous liver tissue

To test the possible involvement of mitochondria in the mechanism by which carnitine inhibited hepatitis, its effect on hepatic mitochondrial functions and mtDNA were examined. As shown in Figure 5, there was a significant decline at 24 weeks and a slight decline at 58 weeks in the RCI, P/O ratio and oxygen consumption of mitochondria from the livers of untreated rats. However, treatment with carnitine significantly inhibited the decline in those indicators of mitochondrial function.

The entire mitochondrial genomes of noncancerous liver tissue from each of the untreated rats at 6, 24 and 58 weeks and carnitine-treated rats at 24 and 58 weeks were analyzed. The points of mtDNA mutations detected were indicated by arrows in Figure 6. The mtDNA in untreated rats of 24 and 58 weeks contained 1 (a T→C transition at nucleotide 2910 in 16S rRNA) and 5 mutations (a T→C transition at nucleotide 2910 in 16S rRNA, a T insertion between 7480 and 7481 in tRNA serine 1, an A→G transition at 12587 in NADH dehydrogenase subunit 5, a T→G transition at 12589 in NADH dehydrogenase subunit 5, a C→G transition at 12878 in NADH dehydrogenase subunit 5), respectively. The liver tissues obtained from carnitine-treated rats at 24 and 58 weeks contained a T→C transition at nucleotide 2910 in 16S rRNA and a C→T transition at nucleotide 8156 in cytochrome c oxidase subunit II, respectively. Compared with the standard rat mtDNA sequence deposited in the Genbank (accession number J01415), we found several common variations (not shown) in all of the samples we analyzed due to the difference of the strains.

ROS generation by mitochondria

To elucidate the mechanism in inhibition of liver injury by carnitine, possible production of ROS by liver mitochondria was tested using L-012 that predominantly reflects the generation of superoxide and hydroxyl radicals.[32] The production of ROS was significantly high at 24 and 58 weeks with no treatment. However, the production of ROS was decreased with carnitine treatment (Fig. 7).

Effects of carnitine on formation of 4-HNE adducts and 8-OHdG in the liver

To test the possible inhibition of oxidative stress by carnitine, its effect on lipid peroxidation and oxidative DNA damage was examined by immunohistochemical staining with anti-4-HNE adducts and anti-8-OHdG antibodies, respectively.

In the livers of 6-week-old LEC rats, no positive immunolabeling of 4-HNE adducts (Fig. 8*a*) or 8-OHdG (Fig. 8*f*) was observed. In contrast, in 24- and 58-week-old untreated rats, patchy immunolabeling of 4-HNE adducts and sporadic expression of 8-OHdG were widely detected in the cytoplasm and nuclei of hepatocytes, respectively (Fig. 8*b*, *d*, *g* and *i*). However, almost no evident occurrence was observed in livers of 24- (Fig. 8*c* and *h*) and 58- (Fig. 8*e* and *j*) week-old carnitine-treated rats.

Effects of carnitine on apoptosis in acute hepatitis phase

We also analyzed the effect of carnitine on apoptosis at the stage of 24 weeks in acute hepatitis. Although the livers from 6-week-old rats showed negligible amounts of TUNEL-positive cells (Fig. 9*a*), the livers from 24-week-old untreated rats showed increased amounts of TUNEL-positive cells (Fig. 9*b*). The treatment of animals with carnitine significantly inhibited the occurrence of TUNEL-positive cells (Fig. 9*c*). No evident occurrence of TUNEL-positive cells was observed in either the untreated or carnitine-treated groups at 58 weeks (data not shown).

Effect of carnitine on preneoplastic liver lesions

To test the possible involvement of mitochondrial protection in hepatocarcinogenesis, the effect of carnitine on the preneoplastic lesions was examined. At age of 6 weeks, no occurrence of GST-P-positive foci was found (Fig. 10*a*). Compared to a untreated group of 58-week-old rats (Fig. 10*b*), there was no evident occurrence of GST-P-positive foci in the carnitine-treated group of 58-week-old rats (Fig. 10*c*). The numbers and the size of GST-P-positive foci per square centimeter in the liver specimens of the carnitine-treated group were significantly lower (Fig. 10*d*) and smaller (Fig. 10*e*) than in those of untreated group, respectively. These results clearly indicate that carnitine decreases the tendency of the occurrence of GST-P-positive preneoplastic lesions in LEC rats. No evident GST-P-positive foci were observed at the 24-week stage.

Effects of carnitine on number of liver cancers in LEC rats

The effect of carnitine on the occurrence of liver tumors in LEC rats was examined. As shown in Figure 11, at the 58-week stage, the number of liver cancers in the untreated group increased significantly, while the number of cancers in the carnitine-treated group markedly decreased.

Discussion

This study demonstrates that free radicals elicited from abnormally accumulated copper induced mtDNA mutation and functional disorder of mitochondria in the livers of LEC rats. This indicated hepatitis and eventually hepatocellular carcinoma, but was inhibited by long-term administration of carnitine.

LEC rats are an animal model of Wilson's disease in humans and are frequently used to clarify the mechanism of hepatocarcinogenesis. Excessive accumulated copper is considered to be responsible for the hepatocarcinogenesis observed in LEC rats.[34] It is now well known that excess heavy metals, such as copper and iron, generate ROS such as superoxide anion and hydrogen peroxide, catalyzing the formation of highly toxic hydroxyl radicals via a Fenton-like reaction.[35] Hydroxyl radicals can then initiate lipid peroxidation chain reactions, thereby disrupting cellular membranes, mainly mitochondrial membranes,[36,37] and damage the DNA of both the mitochondria and nucleus.[3,4]

The mitochondrial genome comprises a 16.5 kb circular double-stranded DNA molecule that encodes 13 polypeptides of the respiratory chain, 22 transfer RNAs and 2 rRNAs required for mitochondrial protein synthesis.[5] Expression of the entire mitochondrial genome is necessary for the maintenance of mitochondrial functions, suggesting that small changes in the sequence of mtDNA might result in profound impairment of such functions. Thus, mitochondrial DNA harboring certain mutations elicited from free radicals might generate abnormal RNAs or proteins in the electron transport system, the latter of which may promote the leakage of electrons from the mitochondrial electron transport chain.[38,39] It has also been well documented that oxidative stress enhances lipid peroxidation and degradation of phospholipids, thereby increasing free forms of fatty acids.[40,41] Because free-form long-chain fatty acids are hydrophobic anions that exhibit properties similar to those of anionic detergents, a rapid increase in their tissue levels might disturb the structure and functions of mitochondrial membranes, the integrity of which is essential to normally functioning mitochondria,[22,42] and the destruction of which would also lead to the leakage of electrons from the mitochondrial electron transport chain.[38,39] Thus, mitochondria might be an important target for copper-induced toxicity and play an important role in the pathogenesis of LEC rats. In fact, several investigations have revealed that there are striking ultrastructural and functional abnormal alterations in the mitochondria of hepatocytes in LEC rats.[16–18] Furthermore, the severity of ultrastructural alterations in the mitochondria correlated with the degree of icterus displayed by the rats.[17] The amounts of endogenously produced free radicals were markedly increased in cells with mutant mtDNA and dysfunctional mitochondria.[9,10] As a result, mitochondria would enter a vicious circle in which the secondary oxidative modification of mitochondria itself is further amplified by increased production of

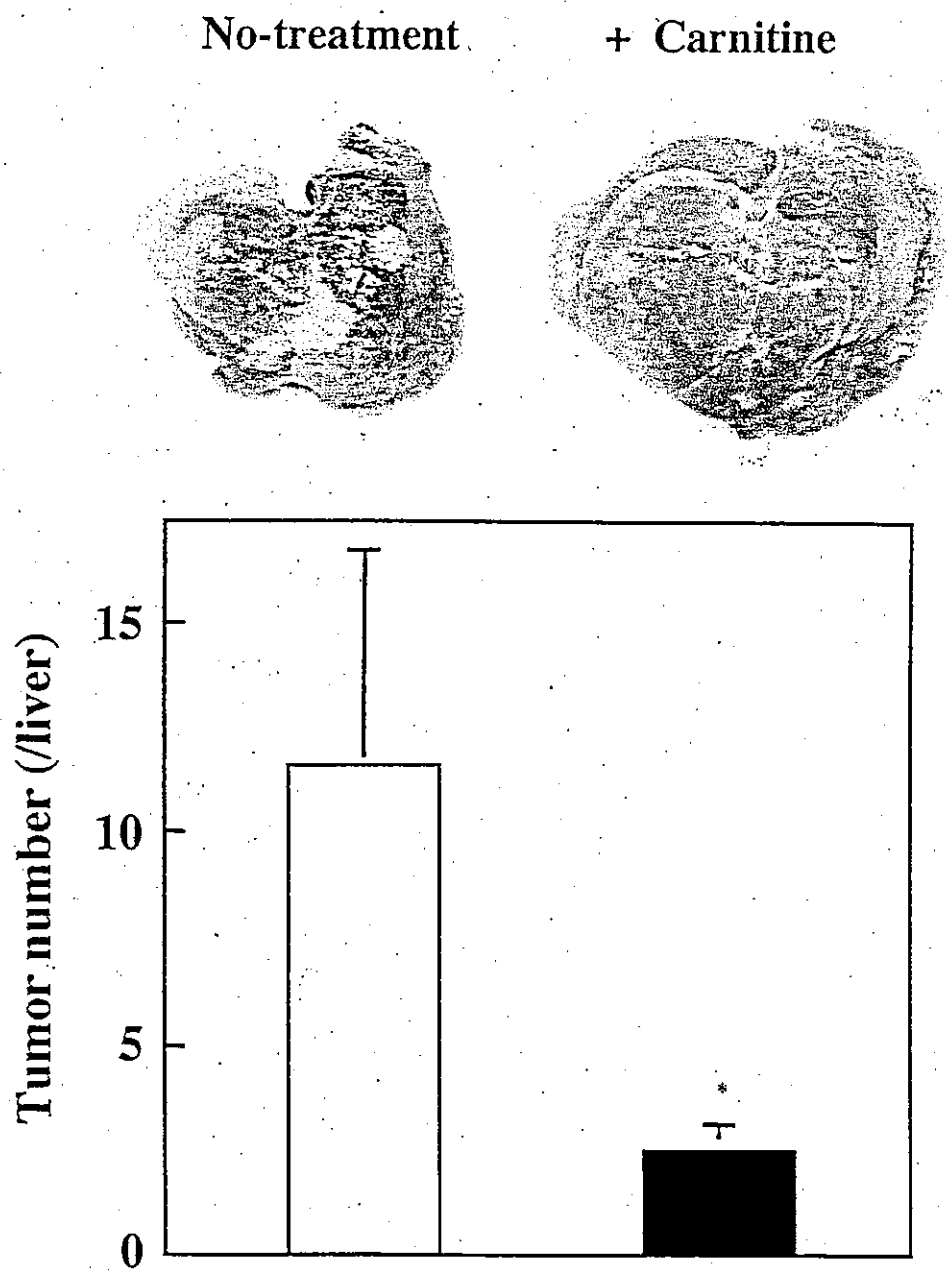

FIGURE 11 – Effects of carnitine on the number of liver tumor. Visible liver cancers large than 1 mm^3 were counted when sacrificed at 58 weeks of age. Open column, untreated group; closed column, carnitine-treated group. Asterisk, $p < 0.01$ *vs.* untreated group.

free radicals. Excessively generated free radicals, therefore, would induce further lipid peroxidation, DNA breakage and the formation of 8-OHdG, which is one of the main DNA modifications induced by ROS.[43,44] The resulting oxidative modification of DNA is considered to contribute to the early stages of hepatocarcinogenesis. In fact, the treatment with carnitine inhibited mitochondrial dysfunction and mtDNA mutation in the early stage of hepatocarcinogenesis. Clinically, our recent report shows that the frequency of mtDNA mutations in both noncancerous and cancerous liver specimens from individuals with hepatocellular carcinoma was markedly increased, and the high rate of mtDNA mutation in cancerous liver tissue is also consistent with the multicentric hepatocarcinogenesis detected clinically.[45]

Cellular levels of carnitine in hepatocytes are determined by *de novo* synthesis and specific carnitine transporters. It has been reported that biosynthesis of carnitine is decreased in rats with liver cirrhosis.[46] The liver is comparatively enriched with transporters to absorb carnitine.[19,20] OCTN-2 is a carnitine transporter located on the plasma membrane, and it might be destroyed when exposed to ROS. In fact, our data show that, in the acute hepatitis phase, the expression of OCTN-2 level is decreased. As a result, disturbance of carnitine absorption might cause transient increase of serum carnitine at 24 weeks. In this case, the administration of carnitine was expected to increase serum carnitine levels significantly because of decreased expression of OCTN-2. Our data also show that carnitine levels in both the serum and liver increased in carnitine-treated animals. The administered carnitine thus facilitates the β-oxidation to generate ATP, thereby minimizing the toxic effects of free form of long-chain fatty acids in and around mitochondria. In fact, mitochondrial dysfunction caused by free fatty acids was inhibited by carnitine.[21,22] Furthermore, carnitine strongly inhibited the destruction of the mitochondrial membrane and mitochondrial dysfunction-induced injury in various types of cells.[22–25] These findings indicate that oxidative stress elicited from abnormally accumulated copper increased the amount of free fatty acids, thereby inducing mitochondrial dysfunction, resulting in cell death and enhanced secondary generation of ROS, which are significantly inhibited by carnitine treatment. Consistent with

this notion is a report indicating that administration of carnitine decreased free fatty acids in serum and tissues and prevented tissue injury in juvenile visceral steatosis (JVS) mice that lack a carnitine transporter.[47,48] It should also be noted that although carnitine lacks activity to scavenge free radicals, this amino acid shows properties similar to that of antioxidants.[26,27] The protective effects of carnitine and its acylesters might reflect their activities to improve energy metabolism and repair oxidized membrane/lipid bilayers,[49,50] thereby suppressing the release of free electrons from mitochondrial electron transport systems, a prerequisite reaction to generate free radicals. The molecular mechanism by which carnitine suppressed hepatic injury and eventual hepatocarcinogenesis should be studied further. It should also be noted that, although carnitine showed potential inhibitory effects on the initiation of hepatocarcinogenesis, it might have the risk of promoting the growth of tumors by facilitating the generation of energy and inhibiting cell death. Thus, clinically, analysis for optimum timing for use is required.

In summary, this study proposes a potential approach to the prevention of hepatocarcinogenesis in an animal model of hepatitis. It is expected that carnitine has good therapeutic potential in individuals with hepatitis.

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Human Molecular Genetics, 2004, Vol. 13, No. 20 *2377–2384*
doi:10.1093/hmg/ddh276
Advance Access published on August 27, 2004

Disease associations and altered immune function in CD45 138G variant carriers

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Received June 28, 2004; Revised August 2, 2004; Accepted August 19, 2004

The CD45 antigen is a haemopoietic cell specific tyrosine phosphatase essential for antigen receptor mediated signalling in lymphocytes. Expression of different patterns of alternatively spliced CD45 isoforms is associated with distinct functions. We recently identified a polymorphism in exon 6 (A138G) of the gene encoding CD45 (*PTPRC*) that results in altered CD45 splicing. The 138G allele is present at a high frequency among Japanese (23.7%), with 5.1% individuals homozygous for the G allele. In this study we show that the A138G polymorphism is the cause of altered CD45 isoform expression, promoting splicing towards low molecular weight CD45 isoforms. We further report that the frequency of A138G heterozygotes is significantly reduced in number in cohorts of patients with autoimmune Graves' disease or hepatitis B infection, whereas G138G homozygotes are absent from a cohort of Hashimoto's thyroiditis patients. We also show that 138G individuals exhibit altered cytokine production *in vitro* and an increased proportion of memory T cells. These data suggest that the 138G variant allele strongly influences these diseases by modulation of immune mechanisms and may have achieved its high frequency as a result of a natural selection probably related to pathogen resistance.

INTRODUCTION

The CD45 (leucocyte common) antigen is a haemopoietic cell specific tyrosine phosphatase essential for antigen receptor mediated signalling in lymphocytes (1,2). Mice and humans lacking CD45 expression are severely immunodeficient. Expression of different patterns of alternatively spliced CD45 isoforms in lymphocytes is associated with distinct functions, but the role of each isoform is unknown (3). In humans, naive T cells express high molecular weight isoforms containing the fourth or A exon ('CD45RA' cells), but following activation the low molecular weight CD45R0 isoform is expressed ('CD45R0' cells). Point mutations in the A exon of CD45 have been suggested to be associated with multiple sclerosis, autoimmune hepatitis, systemic sclerosis and HIV infection in humans (4–8). These polymorphisms (C77G and C59A) prevent the splicing of exon 4, resulting in the presence of an increased proportion of lymphocytes expressing CD45RA-containing isoforms (9,10). Although the C77G variant appears to be associated with certain diseases, the results from these studies are difficult to interpret because of the low frequency of the variant allele (11–14).

We recently identified another polymorphism (A138G) in exon 6 of CD45. The polymorphism results in an amino acid substitution of Thr-47 to Ala in exon 6 and interferes with CD45 alternative splicing (15). In contrast to the C77G variant, peripheral blood T cells from individuals carrying the 138G allele show an increase in the proportion of CD45R0+ cells and a decrease in naive phenotype T cells expressing CD45RA. The 138G allele is present at a high frequency among Koreans (7.3%) and Japanese (23.7%). In Caucasoids, the allele appears to be relatively rare [1.7% in Italians (14) and 0.4% in the UK (15)]. The high allele frequency of this variant in the Japanese population suggests

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a selective advantage for survival, and we have proposed that the changes in CD45 splicing in the 138G carriers might lead to altered immune responses and associations particularly with autoimmune and viral diseases (15). Here we investigate whether the 138G variant is associated with thyroid autoimmune disorders (Hashimoto's thyroiditis and Graves' disease) and viral infections (hepatitis B and hepatitis C). We studied the phenotype and cytokine production of lymphocytes from individuals with the 138G variant and, using a minigene containing the A138G mutation, we examined the effect of the variant on CD45 splicing.

RESULTS

A minigene containing the A138G mutation shows increased splicing of exon 6

We have previously shown by both RT–PCR and flow cytometric analysis that 138G carriers have increased CD45R0 expression in peripheral blood mononuclear cells (PBMCs) (15). We suggested that the mechanism for the altered isoform expression is the A138G substitution in exon 6, as it has been reported previously that changes in this region of exon 6 affect isoform expression, promoting skipping of exon 6 (16). Indeed, Tsai *et al.* (16) have shown that mutations of nucleotides 134–144 at the most 3′ end of exon 6 (LS37 minigene construct) resulted in CD45 mRNA that did not include the exon 6 sequence, regardless of whether it was synthesized in B cells or in thymocytes. In contrast, the control pSV-mini-LCA30 construct could be alternatively spliced, and in EL-4, produced both kinds of transcripts, one including the exon 6 sequence and one without (16) (Fig. 1A and B). In order to examine the role of 138G variant on CD45 splicing, we introduced the 138G mutation into pSV-mini-LCA30 generating the LS138G construct. We then compared the pattern of splicing of the three constructs pSV-mini-LCA30, LS37 and LS138G in Cos-7 cells. Six individual transfections were performed with each of the constructs and the splicing analysed by RT–PCR (Fig. 1A and B).

Consistent with previous studies, the pSV-mini-LCA30 can produce both ex2–6–8 and ex2–8 transcripts in a ratio of 12.1, whereas with LS37 the skipping of exon 6 is significantly increased; almost all the product is ex 2–8, and the ratio is 0.6 (Fig. 1C). Analysis of transcripts derived from the LS138G construct shows an increase in exclusion of exon 6, resulting in more ex 2–8 product and a decrease in the ratio to 6.4.

Taken together, these data provide direct evidence that the 138G mutation is the cause of the altered CD45 phenotype in 138G carriers.

Frequency of the 138G variant in autoimmune and infectious diseases

We studied the frequency of the 138G variant in cohorts of Japanese patients with thyroid autoimmune conditions. In Hashimoto's thyroiditis, cellular and humoral responses to thyroid antigens lead to destruction of the organ and hypothyroidism, whereas Graves' disease (GD) is characterized by hyperthyroidism, caused by stimulatory thyrotropin receptor antibodies. We analysed 126 Hashimoto patients and found 50 A138G heterozygotes (allele frequency, 19.8%), comparable to the frequency in the control population (23.7%) (Table 1). Interestingly no G138G homozygotes were detected amongst the Hashimoto samples, although five were expected according to the Hardy–Weinberg law ($P = 0.02$). We found 31 heterozygotes (frequency 8.9%) and no homozygotes out of the 175 Graves' samples. The difference between the controls and Graves' disease is very significant ($P < 0.001$) and corresponds to a relative risk of 0.44 for the A138 versus the 138G allele. This suggests a dominant effect for the 138G allele in Graves' disease.

We further analysed the frequency of the variant in two important viral infections, hepatitis B and hepatitis C. We found 23 A138G heterozygotes and two homozygotes among 113 hepatitis B samples (allele frequency, 11.95%). The difference between the controls and hepatitis B is significant ($P < 0.005$), corresponding to a relative risk of 0.55 for the A138 versus the 138G allele. In hepatitis C, we found 48 A138G heterozygotes and eight G138G homozygotes in 173 samples, figures that are as expected according to the Hardy–Weinberg law. No significant association of the 138G variant and severity of disease was detected in hepatitis B or hepatitis C. However, studies in larger cohorts of patients are clearly needed in order to establish whether there is any correlation with disease progression. Taken together, these results show a significant protective effect of the 138G allele in Graves' disease and hepatitis B, and a possible recessive effect in Hashimoto's disease.

Phenotypic analysis of PBMC

Because altered immune phenotypes and function are significant features of both animal model and human autoimmune diseases, we next sought evidence for this in individuals carrying the 138G allele. CD45RA and CD45R0 expression defines subsets of CD4 and CD8 cells that have been termed naive and memory cells (17). We have already reported that the proportions of these subsets are altered in individuals carrying the 138G variant allele (15). Here we examined other markers that either distinguished naive and memory cells or the proportions of memory subsets. Our aim was to determine whether only CD45 isoform expression was affected or whether the CD45RA and CD45R0 cells in 138G individuals also exhibited characteristic 'naive' and 'memory' phenotypes. PBMCs from healthy G138G homozygotes, A138G heterozygotes and A138A control homozygotes were analysed by flow cytometry. All the G138G variant samples showed the previously described increased proportion of CD45R0+ T cells (15); among CD8 cells the mean was $49.4 \pm 8.9\%$, when compared with 18.9 ± 9.3 in controls ($P = 0.03$) (Fig. 2A), and in the CD4 subset the mean was 48.4 ± 9.3 versus $32.8 \pm 9.3\%$ in A138A controls. A138G heterozygotes show an intermediate CD45R0+ phenotype for CD8 and CD4 cells (data not shown). Furthermore, G138G individuals exhibit decreased expression of CD27, CD28, CD62L and CCR7 and increased expression of CD11a and CD95 in CD8 cells (Fig. 2B). Less exaggerated changes in expression of these markers were detected in the CD4 cells (Fig. 2C). These changes suggest that the most prominent effect in

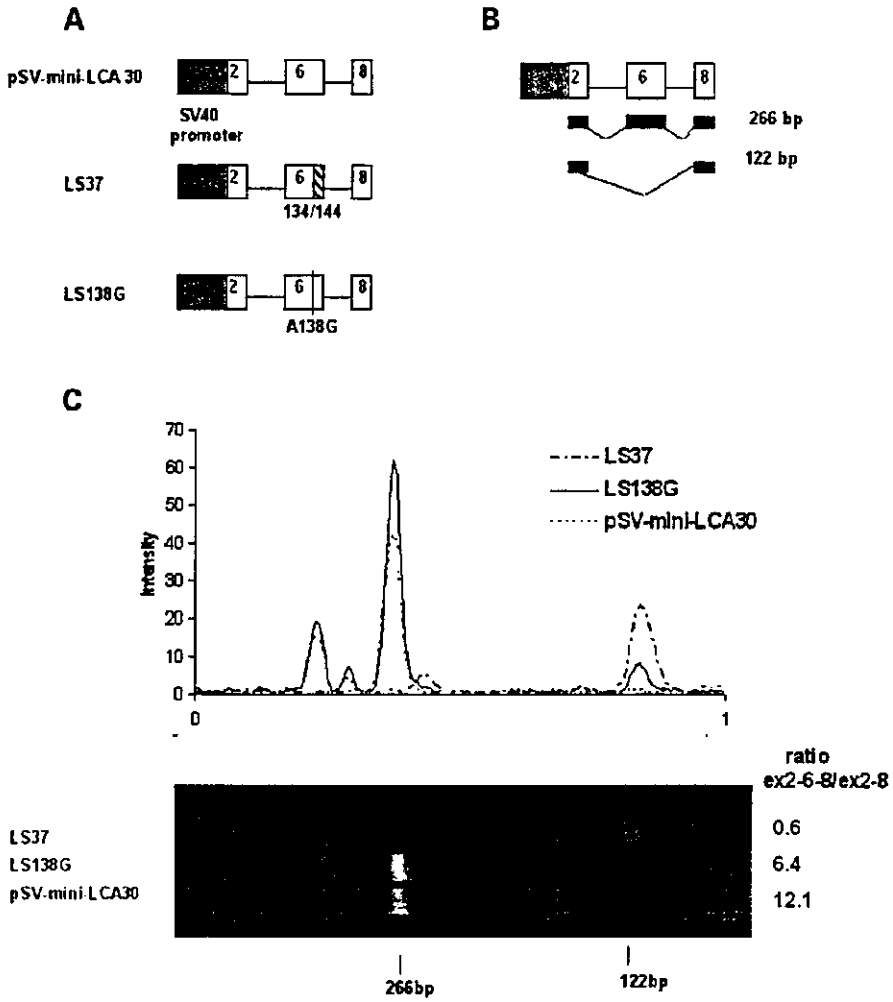

Figure 1. Effect of the exon 6 138G mutation on splicing. (A) Schematic of the minigenes used. The wild-type pSV-mini-LCA30 construct includes variable exon 6 flanked by the constitutive exons 2 and 8. The exons are represented by open boxes and introns are represented by lines. The introns and exons are not drawn to scale. For the mutant version of the minigene LS37, the hatched box within exon 6 represents the 10 bp substitutions made at nucleotides 134–144 at the most 3′ end of the exon. For the mutant LS138G, the line within exon 6 represents the A to G substitution at position 138. (B) The possible alternative splice products are indicated with the expected sizes. (C) RT–PCR analysis of three representative clones expressing the minigenes indicated. Bands in each lane were quantitated and densitograms are shown above the gel, corresponding to the respective minigene splice product. The ratio between the intensity of the ex2–6–8 and ex2–8 products is shown beside the gel and represents the mean result from six transfections for each minigene. A slower migrating band was detected, which represents unspliced RNA, as determined by sequencing.

Table 1. Frequency of CD45 exon 6 A138G alleles in control and disease groups

Disease group	Control	Hashimoto	Graves'	Hepatitis B	Hepatitis C
Total number	176	126	175	113	173
A138A	111	76	144	88	117
A138G (allele frequency %)	65 (23.7%)	50 (19.8%)	31 (9%)**	23 (11.9%)***	48 (19.4%)
G138G	9	0*	0	2	8

Statistically significant differences from controls are indicated by the symbols as follows. *$P = 0.02$ (departure from the Hardy–Weinberg equilibrium with a Chi-square for 2 d.f. of 7.81), **$P < 0.001$ (with a Chi-square of 15.4 for 1 d.f., which corresponds to a relative risk for the heterozygotes of 0.44) and ***$P < 0.005$ (with a Chi-square of 9.3 for 1 d.f., which corresponds to a relative risk of 0.55).

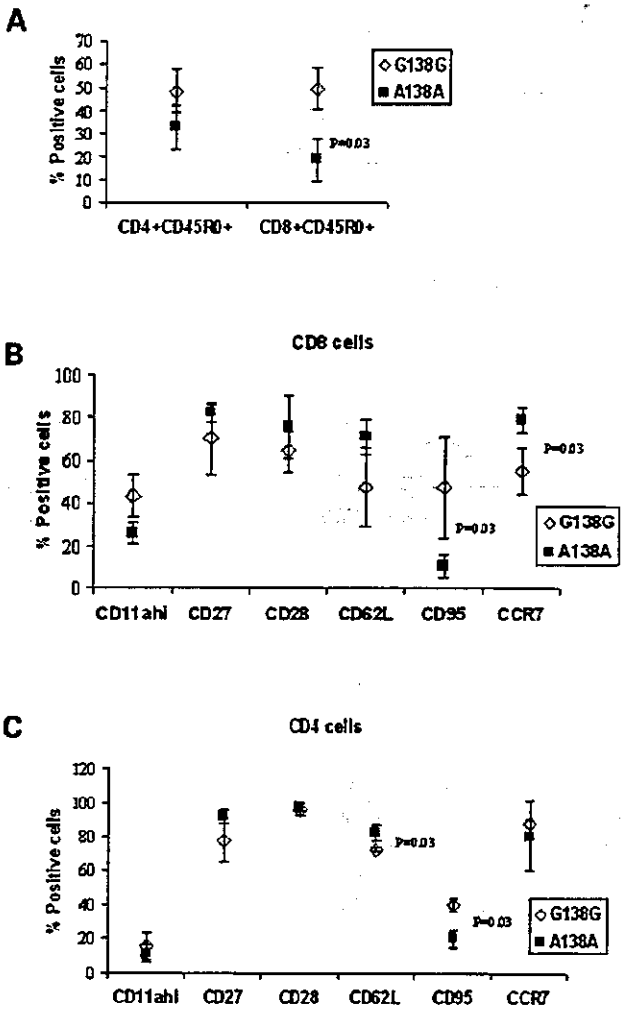

Figure 2. Expression of CD45 isoforms and activation markers on CD4 and CD8 cells from four healthy G138G homozygous and six A138A homozygous control individuals. (A) The proportions of CD8 and CD4 T cells from G138G and A138A control individuals that are CD45R0+. (B) Proportions of CD8 and (C) CD4 T cells that express $CD11a^{hi}$, CD27, CD28, CD62L, CD95 and CCR7. Means and standard deviations of data expressed as the percentage of CD8 and CD4 T cells from four G138G and six A138A control individuals are shown. Four healthy A138G heterozygotes were also assayed in this experiment and showed intermediate phenotypes (data not shown). Differences between G138G and A138A indidviduals for CD8 + CD45R0+, CD8 + CD95+, CD8 + CCR7+ and CD4 + CD62L+, CD4 + CD95+ cells are statistically significant by Mann–Witney, $P = 0.03$.

138G carrying individuals is an increase in the proportion of memory T cells.

Cytokine production by PBMC

We next analysed cytokine production in PBMC from 138G carrying individuals. Intracytoplasmic flow cytometric analysis after stimulation by phorbol myristate (PMA) and ionomycin showed that G138G cells have a significantly higher frequency of intracytoplasmic IFN-gamma positive CD4 and CD8

Table 2. Frequency of IFN-gamma producing CD4 and CD8 T cells of individuals with different 138G alleles

	% CD4 cells expressing IFNγ	% CD8 cells expressing IFNγ
G138G	$30.7 \pm 4.1^{*,a}$	$35.3 \pm 11.6^{+,a}$
A138G	$28.6 \pm 4.3^{**,a}$	$17.8 \pm 4.6^{++,b}$
A138A	18.9 ± 1.8	9.7 ± 2.8

Four individuals of each genotype were studied. Differences between G138G or A138G individuals and A138A controls were analysed by Student's *t*-test. $^{*}P = 0.002$, $^{**}P = 0.006$, $^{+}P = 0.005$, $^{++}P = 0.024$. Differences between 138G individuals and A138A controls were also analysed by Mann–Witney test $^{a}P = 0.012$ and $^{b}P = 0.022$. In this experiment, representative of three similar results, all 12 individuals were analysed in a single experiment to minimize inter-experimental variation.

cells (30.7 versus 18.9% in CD4 and 35.3 versus 9.7% in CD8 cells, Table 2). Heterozygotes showed an intermediate frequency of cytokine-positive cells in both T cell subsets (Table 2 and Fig. 3). Under the conditions of these assays using cryopreserved PBMC, intracytoplasmic staining for cytokines other than IFN-gamma proved unreliable. These preliminary results show that expression of the variant 138G allele is associated with an increased frequency of CD4 and CD8 cells showing intracytoplasmic staining for the Th1 cytokine IFN-gamma. Clearly, further analysis on fresh PBMC for other cytokines will be needed to understand fully the immune function in these individuals.

To exclude the possibility of altered cytokine staining due to the freezing and shipping of the samples, we carried out experiments to compare fresh and frozen cells from six local Chinese and Japanese donors. No differences between fresh and frozen samples were observed in the phenotype of lymphocytes or the frequency of cytokine positive cells. The range for IFN-gamma staining in fresh CD3+ cells was 11.1–21.2% and 10.1–21.4% for frozen cells. Paired results from fresh and frozen samples from each donor were almost identical.

DISCUSSION

In this study we show a protective effect of the 138G variant in cohorts of patients with autoimmune Graves' disease and hepatitis B infection. We provide direct evidence that the A138G mutation is a cause of altered CD45 isoform expression and preliminary evidence that 138G individuals exhibit increased numbers of memory/activated lymphocytes and increased IFN-gamma production.

There are several possible explanations for the effect of the 138G variant. An important factor in the pathogenesis of autoimmune diseases is a change in the balance between Th1 cytokines, which promote cell mediated immunity, and Th2 cytokines, which promote humoral immunity. In Graves' disease, there is a shift towards Th2 cytokine responses (18,19), whereas Hashimoto patients show Th1 activation (20). It is possible that increased IFN-gamma production in 138G carriers would counteract the Th2 cytokine deviation in Graves' disease. Furthermore, it has been

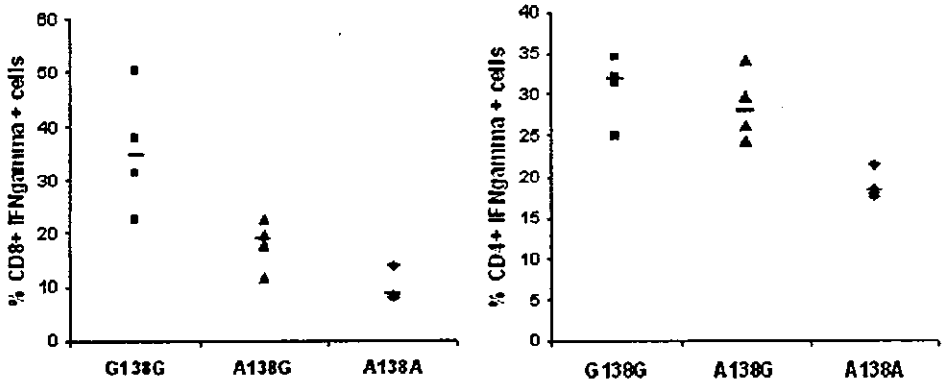

Figure 3. Frequency of intracytoplasmic IFN-gamma positive CD4 or CD8 T cells. PBMC from G138G and A138A homozygous or A138G heterozygous individuals were stained for CD4 or CD8 and for intracytoplasmic IFN-gamma after activation with PMA and ionomycin. Data from 12 individuals analysed in one experiment are shown. Horizontal lines show the mean for each group. Similar results were obtained in a repeat experiment.

suggested that activated (IFN-gamma producing) CD8 cells may reduce the pathogenic Th2 dominance in Graves' disease (19,21). In contrast, increased IFN-gamma production in the 138G variant might not affect the disease course and already polarized Th1 cytokine balance in Hashimoto's thyroiditis. However, the lack of G138G homozygotes in Hashimoto's thyroiditis suggests the possibility of a specific recessive effect in homozygotes, which needs further investigation. Further analysis of immune function in diseased individuals may provide insights into the role of CD45 variant alleles in protection against disease or disease pathogenesis.

The contribution of CD8 cells to the control of hepatitis B virus infection is well documented (22). In addition to clearance of infected cells by cytolytic CD8 cells, the anti-viral effect of IFN-gamma produced by these cells has been shown to be an important protective mechanism (23). It is very likely that the increased proportions of activated T cells and IFN-gamma production in A138G neonates would limit amplification of the virus. Furthermore, it has been suggested that neonates have Th2 biased immune responses (24,25). It is therefore possible that the prevalence of Th1 cytokines in A138G infants would be beneficial at this early stage of life in controlling hepatitis B infection, whereas it would not have such a significant impact later in life. This might be the case for the hepatitis C cohort we have studied. Whatever the mechanism, comparisons of immune responses of individuals carrying or lacking the 138G allele may provide insights into the molecular mechanisms underlying the interactions between hepatitis B and hepatitis C viruses and IFN-gamma. Clearly, further independent cohort studies, family based approaches and immune functional studies in normal individuals and patients, need to be performed to confirm our preliminary functional data and elucidate the role of this polymorphism in disease incidence and progression.

The expression of CD45 is essential for normal development and function of lymphocytes. Both mice and humans lacking CD45 expression are severely immunodeficient (26–29). However, notwithstanding the clear evidence that CD45 expression is essential for normal lymphocyte function and that N-terminal alternative splicing is conserved in fish, birds and mammals (30,31), there is no clear understanding of the role of CD45 isoforms in lymphocyte function. In mice, a point mutation in the cytoplasmic domain has been associated with a lupus-like autoimmune disease (32) and the authors postulated that this was due to an effect on dimerization of CD45 molecules. Others have shown that a large extracellular domain is required for TcR signalling in transfected cell lines (33). On the other hand, evidence from transgenic mice expressing single CD45 isoforms suggests that different isoforms reconstitute immune function equally well if they are equally well expressed (34). Here we show that a relatively subtle alteration in alternative splicing is associated with altered disease susceptibility and increased IFN-gamma production. The mechanism of these effects is not known, but CD45 has been reported to affect cytokine signalling pathways (35). We speculate therefore that specific combinations, rather than individual CD45 isoforms, may have the most profound effect on signalling. In 138G individuals, excess expression of low molecular weight CD45 isoforms may lead to altered signalling and increased IFN-gamma production. This in turn may affect disease susceptibility.

There have been previous reports of altered CD45 isoform expression in disease (36–38), but we describe here what we believe is evidence that genetic variants affecting CD45 isoform expression are associated with autommunity and viral infection. Although one possibility is that the effect of the 138G allele is neutral, the most likely explanation for the high frequency of this variant is natural selection. In contrast to this allele, most neutral polymorphic variants are at a lower frequency and are changes in third base pair positions or in introns. Given the functional effect and high frequency of the 138G variant not only in Japanese but also in China (unpublished data) and Korea (15), natural selection is therefore the most likely explanation for its frequency. The original selection for the 138G CD45 variant may have been with respect to pathogen resistance and what we see now is a residue of this after the pathogen effect has gone. The high frequency of 138G carrying individuals (~40% in Japan) suggests that the presence of allele may also affect susceptibility and pathogenesis to other autoimmune and infectious diseases.

MATERIALS AND METHODS

Materials

DNA samples from 175 Graves' and 126 Hashimoto patients were obtained through the Osaka City University Hospital. Hyperthyroidism due to Graves' disease was diagnosed on the basis of history and signs of hyperthyroidism with diffuse goiter and the laboratory findings, including elevated serum-free T4 and T3 concentrations, undetectable serum thyroid stimulating hormone (TSH) and positive TSH receptor antibody. Hashimoto's thyroiditis was diagnosed by positive thyroglobulin and/or thyroid peroxidase antibodies, reduced echogenicity on thyroid ultrasound and normal or elevated TSH level. In total, 113 hepatitis B and 173 hepatitis C samples were collected in the outpatient clinic of Osaka City University Hospital. All of the 113 hepatitis B patients were infected at birth by transmission from their mothers and were positive for hepatitis B surface antigen. Of the hepatitis B patients 22% were carriers (alanine aminotransferase activity within normal range), 58% had chronic hepatitis (elevation of alanine aminotransferase for more than 6 months and liver inflammation histologically proven by tissue biopsy), 16% had liver cirhosis and 4% hepatocellular carcinoma. The hepatitis C patients were infected later in life and were all positive for antibodies to HCV antigen. In all samples, HCV RNA was detected, except for four patients who had cleared the virus (two of these were A138G heterozygous and one G138G homozygous). In the hepatitis C group of patients, 13% were carriers, 58% had chronic hepatitis, 16% liver cirhosis, 10% hepatocellular carcinoma and 3% had cleared the virus. Control genomic DNA samples from 176 Japanese, collected from Osaka City University Medical School, have been described previously (15). All of the control and patient groups studied are of similar ethnic origin and live in the area surrounding the Osaka University Hospital. Approval was obtained from the ethical committee of the City University Graduate School of Medicine Osaka and the patients gave consent for the study.

Amplification refractory mutation system PCR

To detect carriers of the exon 6 A138G mutations, we used the amplification refractory mutation system (ARMS) PCR with two separate reaction mixes, containing one forward primer and one of the two reverse primers as previously described (15). The presence of the 138G variant allele in all of the samples was confirmed by sequencing.

Flow cytometric analysis

Surface phenotypic analysis and intracytoplasmic staining for IFN-gamma were performed on cryopreserved PBMC (shipped from Japan to the UK) from six healthy A138A controls and four healthy G138G homozygous carriers. Cells from four A138G heterozygotes were also analysed (data not shown). The ages of all subjects in this study were between 27 and 58 years. In total, 2×10^5 PBMC were stained with either allophycocyanine (APC)-conjugated CD4 (S3.3, Caltag, Silverstone, UK) or CD8–APC (clone RPA/T8, Pharmingen, San Diego, CA, USA) along with fluorescein isothyocyanate (FITC)-conjugated CD45RA (clone HI100, Pharmingen) and phycoerythrin (PE)-conjugated CD45R0 (clone UCHL1, Pharmingen) mAbs in a single step at 4°C for 20 min and washed with PBS, containing 0.2% BSA. The following reagents and antibodies were also used to stain cell suspensions: CD11a–FITC (G43-25B), CD28–FITC (CD28.2), CD62L–FITC (Dreg56), CD95–FITC (DX2), CCR7 (2H4), all from Pharmingen; CD27–FITC (LT27), from Serotec (Kidlington, UK).

For intracytoplasmic staining 1×10^5 PBMC per well were incubated in U-bottom 96-well plates in 200 μl of RPMI1640 + 10% FCS in the presence of 50 ng/ml PMA and 0.5 μg/ml ionomycin. GolgiPlug (Pharmingen) was added after 2 h and cells incubated for an additional 12 h. The cells were surface labelled with CD4–APC or CD8–APC antibodies as described earlier and permeabilized with 40 μl Permafix (OrthoDiagnostic, UK) for 40 min in the dark. The cells were washed and stained with FITC conjugated IFN-gamma antibody (clone 25723.11, Pharmingen) for 30 min at room temperature. Isotype matched mAbs were used as controls. In total, 10 000 or 50 000 events per sample were collected on a FACSCalibur (Becton Dickinson, Mountain View, CA, USA) and analysed using WinMDI software.

A comparison of lymphocyte phenotype and the frequency of intracytoplasmic IFN-gamma positive cells in fresh and cryopreserved PBMC was performed on six local Chinese and Japanese donors.

Statistical analysis

Chi-square test, using Yates continuity correction to allow for small numbers, was used to analyse the disease association of the 138G variant allele. For comparison of phenotypic analyses between cell subsets in A138G and control individuals, Student's *t*-test assuming equal variance was used. In addition, we have also reanalysed the data using the non-parametric Mann–Witney test, because the data may not be normally distributed.

Minigene constructs

The minigene pSV-mini-LCA30 has been described previously (16) (kindly provided by H. Saito, Dana Faber Cancer Institute, Boston, MA, USA) and contains exons 2, 6 and 8 under the control of the SV40 early promoter (Fig. 1A). It has been demonstrated that the pSV-mini-LCA30 can be alternatively spliced to produce two kinds of transcripts, one including the exon 6 sequence and one without (Fig. 1B). The LS37 minigene was also used (Fig. 1A) and contains mutation of nucleotides 134–144 at the most 3′ end of exon 6, which have been shown to result in CD45 mRNA that did not include the exon 6 sequence (16).

To study the effect of the 138G mutation on splicing, the 138G substitution was introduced into the pSV-mini-LCA30 construct according to the QuickChange site-directed mutagenesis kit (Stratagene) generating construct LS138G. Clones containing the 138G mutation were identified by sequencing. Exponentially growing Cos-7 cells (5×10^6 cells) were mixed with 1 μg of the linearized (*Pvu*I digested) respective

constructs in PBS, then subjected to electroporation (Bio-Rad Gene Pulser II, Bio-Rad, Hemel Hempsted, UK) at 25 μF and 280 V. After 48 h cells were harvested and resuspended in TRI-reagent (Sigma) for RNA extraction and RT–PCR analysis as described subsequently. Six independent transfections were performed and analysed for each minigene.

RT–PCR analysis

Total RNA was extracted from the transfected cells and cDNA synthesis performed using First Strand cDNA Synthesis Kit (Amersham Biosciences). The cDNA was amplified by primers in exon 2 (ex 2 fw: 5′-TAGGGACACGGCTGGCTT CCAG-3′) and exon 8 (ex 8 rev: 5′-CATGTTGGCTTAGAT GGAGTAG-3′) generating bands for the ex 2–6–8 product of 266 bp and for the ex 2–8 of 122 bp (Fig. 1B). The PCR conditions for amplification included a 4 min incubation at 94°C followed by 25 reaction cycles (1 min at 94°C, 1 min at 55°C, 1 min at 72°C) and final 10 min extension at 72°C. The identity of the RT–PCR products was confirmed by sequencing. RT–PCR products were resolved on Visigel Separation Matrix (Stratagene) and the bands quantitated using Quality One software (Bio-Rad).

ACKNOWLEDGEMENTS

We thank Dr Persephone Borrow for helpful discussions and suggestions, and Dr H. Saito for the CD45 LS37 and pSV-mini-LCA30 minigenes.

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J Gastroenterol 2004; 39:1063–1068
DOI 10.1007/s00535-004-1445-3

Journal of Gastroenterology

Correlation between clinical characteristics and mitochondrial D-loop DNA mutations in hepatocellular carcinoma

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Background. Mitochondrial DNA (mtDNA) mutations are found in many kinds of human cancer. The aim of this study was to evaluate the relationship between mtDNA mutations in the liver and human hepatocarcinogenesis. *Methods.* Direct sequencing of mtDNA was done in 54 hepatocellular carcinomas (HCCs) and 47 surrounding liver tissue samples, obtained from 54 patients with HCC, and in 5 liver samples without inflammation, obtained from 5 patients with metastatic liver tumors. We also examined *p53* mutations in the 54 HCCs to examine the correlation between nuclear DNA mutations and mtDNA mutations. *Results.* Mutations of mtDNA in the D-loop region were found in both HCC and noncancerous liver tissue. In normal liver without chronic inflammation, no mtDNA mutation was detected. In every case, the number of mtDNA mutations in HCC correlated with that in noncancerous liver tissue. Twelve of 52 mutation sites in the D-loop region of mtDNA were specific for HCC. The mean number of mtDNA mutations was 1.7 in well-differentiated HCC, as compared with 4.5 in moderately differentiated HCC and 4.6 in poorly differentiated HCC. The frequency of mtDNA mutations was thus higher in less differentiated HCC. We detected *p53* mutations in 15 (28%) of 54 HCCs. The mean number of mtDNA mutations was 5.3 in HCC with *p53* mutations and 3.8 in HCC with wild-type *p53* ($P = 0.024$). *Conclusions.* A higher frequency of mtDNA mutations was found in less differentiated HCCs, and it is also possible that mtDNA mutations are related to nuclear DNA mutations in HCC. The accumulation of mtDNA mutations is a useful predictor of hepatocarcinogenesis.

Key words: hepatocellular carcinoma, mitochondrial DNA, *p53*, reactive oxygen species

Received: December 1, 2003 / Accepted: April 16, 2004
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Introduction

Hepatocellular carcinoma (HCC) is one of the most lethal tumors worldwide.[1] Most patients with HCC have been infected with hepatitis B virus or hepatitis C virus.[2] Unlike other types of cancer, HCC is usually preceded by 20 to 40 years of chronic infection. Normalization of the serum activity of alanine aminotransferase by treatment with interferon reduces the rate of hepatocarcinogenesis, even if hepatitis virus is not eliminated.[3,4] Chronic viral inflammation is associated with repeated hepatocyte necrosis, followed by regeneration. Such an accelerated cell cycle may be associated with the accumulation of genetic errors in the liver. It is not clear which genetic change is most important in HCC, although many studies have examined hepatocarcinogenesis in humans and in animal models.[5–8]

Some studies have suggested that reactive oxygen species (ROS) are one of the most important factors in chronic inflammation-related carcinogenesis.[9] ROS, which increase in the liver during chronic viral hepatitis, injure mitochondrial DNA (mtDNA) more frequently than nuclear DNA, because mitochondria lack histones, which protect against ROS-induced DNA injury. Recently, mtDNA mutations have been detected in human colorectal,[10] ovarian,[11] and thyroid cancers,[12] as well as in HCC.[13] Most mutations in cancers occur in the D-loop region of mtDNA. There are two hypervariable sites in this region. Stoneking[14] reported that these hypervariable sites may be mutation hotspots in mtDNA for germline and somatic mutations, including those associated with cancer. The D-loop region functions as a promoter for both the heavy and light strands of mtDNA, and alterations of this region may influence the rate of mtDNA replication. Accumulating evidence suggests that mtDNA mutations are useful markers for diagnosis, metastasis, and prognosis. Also, polymorphism of mtDNA is well known, and mtDNA mutations occur during the process of senescence.[15]

Table 1. Clinicopathologic features of patients

Variable	Patients with HCC (n = 54)
Age (years)[a]	61 ± 8
Male, Female	47, 7
Serum virus markers	
HBsAg-postive	13
Anti-HCV-positive	30
Both negative	11
Both positive	0
Tumor size (mm)[a]	39 ± 25
Tumor histologic findings	
Well-differentiated HCC	6
Moderately differentiated HCC	26
Poorly differentiated HCC	22
Cirrhosis in noncancerous tissue	
Present, absent	24, 23

[a]Results are shown as means ± SD

It remains unclear whether mtDNA mutations can induce neoplasms. To study the role of mtDNA mutations in hepatocarcinogenesis, we examined not only mitochondrial D-loop mutations but also *p53* tumor suppressor gene mutations in human HCC. In addition, we contrasted mtDNA mutations with the clinical characteristics of HCC.

Patients and methods

Tissue specimens

We obtained liver specimens, after obtaining informed consent, from 54 consecutive Japanese patients with HCC who underwent surgery from 1997 through 2001 at Osaka City University Graduate School of Medicine (Table 1). The specimens included 30 HCC samples and 26 corresponding noncancerous liver samples with chronic hepatitis C virus (HCV) infection; 13 HCC samples and 10 corresponding noncancerous liver samples with chronic hepatitis B virus (HBV) infection; and 11 HCC samples and 11 corresponding noncancerous liver samples without viral infection. HCC was diagnosed histologically as well-differentiated, moderately differentiated, or poorly differentiated in 6, 26, and 22 patients, respectively. Liver specimens without inflammation were obtained from 5 patients with metastatic liver tumor. The study was performed in accordance with the Helsinki Declaration of 1975 (1983 revision) and was approved by the Ethics Committee of Osaka City University Graduate School of Medicine.

DNA extraction from tissue

Liver specimens were immediately frozen and kept at −80°C until extraction of DNA. Total cellular DNA was extracted from tumorous and corresponding noncancerous tissue by the phenol-chloroform and ethanol precipitation method, as described previously.[13]

Sequencing of the D-loop region of mitochondrial DNA

Each DNA sample (50 ng) was subjected to amplification by polymerase chain reaction (PCR), with the use of overlapping sets of primers to screen the entire mitochondrial genome. PCR (initial incubation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min) was performed in a final volume of 50 µl, with a GeneAmp PCR system 9600 (Perkin-Elmer Life Sciences Japan, Tokyo, Japan). The generation of large PCR products excluded the possibility of nuclear pseudogenes complicating the analysis.[16] In addition, the primers were selected by analysis of cell lines devoid of mtDNA to avoid coamplification. Aberrant PCR products were purified with a QIAquick PCR purification kit (Qiagen, Tokyo, Japan) and sequenced with an Applied Biosystems DNA sequencer (Perkin-Elmer) and a Dye Terminator Cycle Sequencing FS Ready Reaction kit (Applied Biosystems, Tokyo, Japan). The sequence of the displacement (D)-loop (nucleotides 100-600) was examined for the 5 control subjects and 54 patients with HCC. All mutations were confirmed by repeated analysis of DNA extracted from the tissue samples.

Sequencing of p53

We examined exons 5 through 8 of the *p53* genes (in which 98% of *p53* mutations had occurred) of the 54 tumors by direct sequencing, as described previously.[17] In brief, 100 ng of genomic DNA was subjected to 35

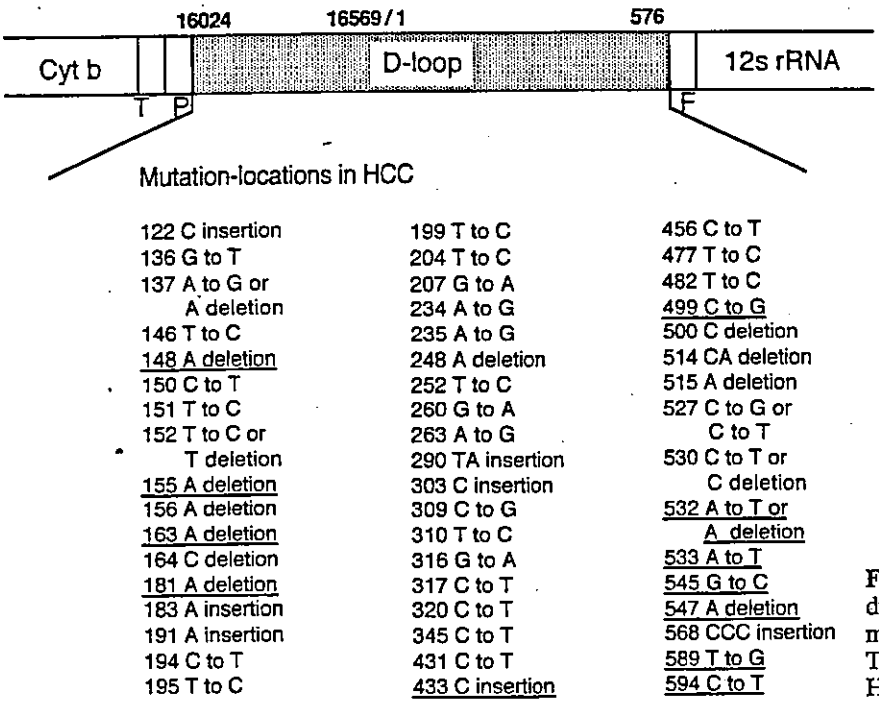

Fig. 1. Scheme of the D-loop of mitochondria. Fifty-two locations in the D-loop were mutated in hepatocellular carcinoma (*HCC*). The *underlined* mutations were found only in HCC, and not in noncancerous liver

PCR cycles (94°C, 55°C, 72°C for 0.5, 0.5, and 1 min, respectively) with rTaq DNA polymerase (Takara Shuzo, Otsu, Japan). The PCR products were treated with exonuclease and alkaline phosphatase. Double-stranded DNA was sequenced by the dideoxy method with fluorescently labeled 2′, 3′-dideoxynucleoside 5′-triphosphates and each primer. Gel electrophoresis and DNA sequencing were performed with a DNA sequencing system (373A; Applied Biosystems).

Statistics

Student's *t*-test was used to analyze the statistical significance of differences in age, hepatitis B surface antigen (HBsAg), hepatitis C virus antibody (HCVAb), tumor size, portal invasion, intrahepatic metastasis, cirrhosis, and histological findings. The significance of differences between median values was evaluated by the Mann-Whitney *U*-test. *P* values of less than 0.05 were considered to indicate statistical significance.

Results

Mutations of mtDNA were found in both HCC and noncancerous liver tissue (Fig. 1). Previously, three mutation sites in mtDNA have been reported to be unique for Japanese. We evaluated our results after excluding these sites. The mean number of mtDNA mutations was 4.4 in HCC and 3.4 in noncancerous liver tissue ($P = 0.03$). In every case, the number of mtDNA mutations in HCC correlated with that in noncancerous liver tissue (Fig. 2). In brief, mtDNA mutations in HCC were similar to those in the corresponding noncancerous liver tissue sample, or, more mutation sites in mtDNA were found in HCC, except in 4 patients. Twelve mutation sites were found in the D-loop region of mtDNA from HCC, but not in noncancerous tissue.

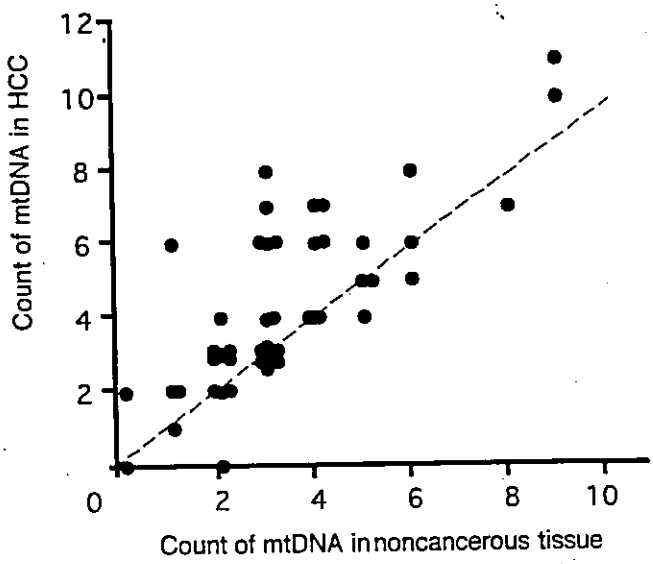

Fig. 2. Correlation between the number of mitochondrial (mt)DNA mutations in HCC and that in noncancerous liver. In 33 patients, mtDNA mutations were more frequently detected in HCC than in the corresponding noncancerous liver samples. However, in 4 patients, mtDNA mutations were more frequent in noncancerous liver

Table 2. Relationship between mtDNA mutations and clinical background

	n	Count of mtDNA mutations	*P*	
HCC				
Virus markers				
HBV	13	3.6 ± 2.4	0.51	
HCV	30	4.1 ± 2.1		0.14
Non-B, non-C	11	5.1 ± 2.6	0.18	
Tumor size (cm)				
<3	22	4.4 ± 2.5	0.59	
≥3	32	4.1 ± 2.2		
Differentiation				
Well	6	1.7 ± 1.4	0.01*	
Moderately	26	4.5 ± 2.2		0.01*
Poorly	22	4.6 ± 2.3	0.84	
Portal tumor thrombus				
Negative	36	3.8 ± 2.1	0.16	
Positive	18	4.8 ± 2.7		
Intrahepatic metastasis				
Negative	32	3.9 ± 2.4	0.31	
Positive	22	4.6 ± 2.2		
p53 Mutation				
Negative	39	3.7 ± 2.2	0.02*	
Positive	15	5.3 ± 2.2		
Noncancerous tissue				
Age (years)				
<60	22	3.1 ± 1.8	0.56	
≥60	25	3.6 ± 2.3		
Virus markers				
HBV	10	3.1 ± 1.6	0.98	
HCV	26	3.1 ± 2.1		0.14
Non-B, non-C	11	4.5 ± 2.3	0.09	
Cirrhosis				
Negative	23	4.0 ± 2.6	0.07	
Positive	24	2.9 ± 1.5		
Inflammation				
Mild	12	3.1 ± 1.7	0.71	
Moderate	26	3.4 ± 2.1		0.63
Severe	9	3.7 ± 3.0	0.79	

*Statistically significant

Nine of these D-loop sites were found in different patients. Mutations of the D-loop were identified at nucleotide 547 in 2 patients, nucleotide 532 in 3 patients, and nucleotide 594 in 5 patients. The mean number of mtDNA mutations was 1.7 in well-differentiated HCC, as compared with 4.5 in moderately differentiated HCC, and 4.6 in poorly differentiated HCC. Less differentiated HCCs thus had a higher frequency of mtDNA mutations (Table 2). The mean number of mtDNA mutations was 4.8 in HCC with portal tumor thrombus and 3.8 in HCC without it. The average number of mtDNA mutations was 4.6 in HCC with intrahepatic metastasis and 3.9 in HCC without it. We detected *p53* mutations in 15 (28%) of the 54 HCCs (Table 3). The mean number of mtDNA mutations in HCC with *p53* mutations (5.3) was significantly higher than that in HCC with wild-type *p53* (3.8; $P = 0.024$).

The surrounding liver tissue samples were classified into three groups on the basis of pathological fibrous changes. The mean number of mtDNA mutations was 3 in liver tissue with mild fibrous changes, 4.6 in liver tissue with moderate fibrous changes, and 2.9 in liver tissue with cirrhosis. Next, these liver tissues were classified into three groups according to the degree of inflammatory change. The mean number of mtDNA mutations was 3.2 in liver tissue with mild inflammatory changes, 3.4 in liver with moderate inflammatory changes, and 3.7 in liver with severe inflammatory changes. In the five normal livers without inflammation, no mtDNA mutation was detected. The mean number of mtDNA mutations was 3.1 in liver tissue with HBV, 3.1 in liver tissue with HCV, and 4.5 in liver tissue without hepatitis virus. The mean number of mtDNA mutations was 3.6 in liver tissue samples from

Table 3. *p53* mutations in HCC

Patient no.	Histology (differentiation)	Count of mtDNA mutations	*p53* Mutation
19	Moderate	3	Codon 220, TAT to TGT (Tyr to Cys)
92	Moderate	3	Codon 155, ACC to AAC (Thr to Asn)
46	Moderate	3	Codon 225, TCT to TTT (Ser to Phe)
104	Moderate	4	Codon 221, GAG to GGG (Glu to Gly)
8	Moderate	5	Codon 132, AAG to TTG (Lys to Leu)
80	Moderate	5	Codon 272, GAG to GTG (Gly to Val)
34	Moderate	6	Exon 5 insertion
171	Moderate	6	Codon 184, GAT to GTT (Asp to Tyr)
12	Moderate	8	Codon 159, GCC to AGC (Ala to Ser)
27	Poor	4	Exon 8 insertion
59	Poor	4	Codon 189, GCC to GTC (Ala to Val)
174	Poor	5	Codon 184, GAT to GTT (vAsp to Tyr)
16	Poor	5	Codon 262, GGT to GTT (Gly to Val)
47	Poor	8	Codon 211, ACT to ATT (Thr to Ile)
40	Poor	11	Codon 133, ATG to TTG (Met to Leu)

patients 60 years or older and 3.1 in liver tissue samples from patients younger than 60 years (Table 2).

Discussion

The human mitochondrial genome comprises a 16.5-kb circular double-stranded DNA molecule that encodes 13 polypeptides of the respiratory chain, 22 transfer RNAs, and 2 rRNAs required for protein synthesis. Expression of the entire mitochondrial genome is necessary for the maintenance of mitochondrial functions, suggesting that small changes in the sequence of mtDNA may markedly impair such functions. Our study showed that mtDNA was more frequently mutated in HCC than in noncancerous liver tissue. Inherited mutations in mtDNA are associated with mitochondrial myopathy, encephalopathy, and diabetes mellitus.[18] However, patients with these conditions have not been reported to be at increased risk for cancer. How mutations in mtDNA are related to carcinogenesis remains elusive. A recent study reported that depletion of mtDNA in a breast cancer cell line resulted in alteration of nuclear gene expression, which plays an important role in cell growth, differentiation, and apoptosis.[19] Habano et al.[20] have shown that mtDNA mutations are related to nuclear microsatellite instability in intestinal-type gastric cancer. We found that mtDNA mutations were significantly more frequent in HCC with *p53* mutations than in HCC with wild-type *p53*. In addition, mtDNA mutations were more frequently detected in less differentiated types of HCC. These findings suggest that mtDNA mutations may be related to nuclear DNA mutations and lead to dedifferentiated HCC. On the other hand, the presence of portal thrombus and of intrahepatic metastasis was unrelated to the mean number of mtDNA mutations in HCC. Future studies should examine the relationship of mtDNA mutations to recurrence and survival rates in HCC.

Our study demonstrated the presence of mtDNA mutations in noncancerous liver tissue that had histopathological evidence of chronic liver disease, including cirrhosis. A few of the mtDNA mutations detected in the present study may be polymorphisms. Studies of cancer arising in other organs found no mtDNA mutations in noncancerous tissue.[10] Muller-Hocker et al.[21] detected impairment of the respiratory chain in 50 (78%) of 64 cirrhotic livers, with a particularly high prevalence in patients older than 50 years. Our results are consistent with these findings, excluding the relationship to age. We found no relationship between the presence of mtDNA mutations and the age of patients with HCC. Moreover, there was no discernible relationship between hepatitis virus infection and mtDNA mutations in noncancerous liver or between the type of hepatitis virus and mtDNA mutations. Our findings suggested that ROS, irrespective of their underlying source, injured mtDNA in the liver. The mean number of mtDNA mutations was higher in liver samples with evidence of active inflammation. Chronic inflammation may thus increase mtDNA mutations in precancerous liver at random.

We found several mtDNA mutations in the D-loop that are specific for HCC. However, these mutations are not common in HCC. Okochi et al.[22] have reported that mtDNA mutations in serum are useful clinical markers of HCC. Whether such mutations are specific for cancer remains doubtful. It is unclear which

mutation in mtDNA is important for carcinogenesis.[23] Ha et al.[24] suggested that cells with mitochondrial C-tract alteration may have some selective growth advantage in premalignant lesions of the head and neck. In experimental models, cells of mice with designed mutations in mtDNA had a unique phenotype, unrelated to cancer.[25] In contrast, mtDNA is randomly mutated in chronic liver diseases. When the transfer RNA coding region of mtDNA is mutated, hepatocytes undergo apoptosis before cancer develops.[26] When mutations occur in other regions of mtDNA, hepatocytes survive, and mtDNA mutations accumulate. Nuclear DNA is most likely unstable in such hepatocytes. Our data suggest that the accumulation of mtDNA mutations is a more important marker for the development of cancer than the specific locations of such mutations. Future studies must examine the full length of mtDNA to identify HCC-specific mutations.

In conclusion, our results suggest that mtDNA mutations resulting from mitochondrial injury by ROS accumulate in liver tissue affected by chronic inflammatory disease. In such tissue, nuclear DNA is also injured and mutated, leading to carcinogenesis. The accumulation of mtDNA mutations is thus a useful predictor of hepatocarcinogenesis.

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JOURNAL OF GASTROENTEROLOGY

INDIAN SOCIETY OF GASTROENTEROLOGY

34

Indian Journal of Gastroenterology

Volume 23 Number 5 September - October 2004

In this issue....

w.indianjgastro.com ISSN 0254-8860

Official Journal of the Indian Society of Gastroenterology

to convey exactly this meaning, i.e., whereas the 'do nothing' approach may appear cheaper, it would not achieve the necessary objective and hence was not worthy of comparison or consideration.

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Acute appendicitis in left scrotum

We read the article by Sharma *et al*[1] about the occurrence of acute appendicular perforation in a right inguinal hernial sac. This entity has been described earlier in children. In fact, the first recorded appendicectomy in 1736 was performed by Claudius Amyand, surgeon to King George II, on an 11-year-old who had a perforated appendix within an inguinal hernia.[2] The term Amyand's hernia has been used variously to refer to occurrence of an inflamed appendix within an inguinal hernia,[3] a perforated appendix within an inguinal hernia,[2] or a non-inflamed appendix within an irreducible inguinal hernia.

A 35-year-old man presented with left inguinoscrotal swelling since two months, and acute pain in the left scrotum associated with vomiting since one day. Clinical examination revealed tachycardia with tender left inguinoscrotal swelling. A clinical diagnosis of strangulated inguinal hernia was made. Operative findings included inflamed appendix with inflammatory fluid in the left hernial sac. The terminal ileum and cecum were minimally congested. Appendicectomy was done with exploration of the small bowel, which was normal. Modified Bassini's herniorraphy was done. Postoperative recovery was uneventful. Barium study after six weeks ruled out situs inversus.

The incidence of appendicitis within an incarcerated hernia is 0.13%.[4] A majority of these are in children and in right-sided inguinal hernia. Appendix in left inguinal hernia, and its presentation in adults, is rare. Presence of cecum and appendix in the left inguinal hernia is seen in situs inversus, intestinal malrotation or mobile cecum.

At surgery, if the peritoneal cavity is uncontaminated it must be protected from contamination. Introducing a foreign material into a contaminated field has its dangers. It has been recommended that repair be done without using a synthetic mesh.[3]

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Gastric ulcer detected incidentally by renal scintigraphy

Technetium-99m labeled RBCs and diethylene-triamine-penta acetic acid (DTPA) human serum albumin (HAS-D) are widely used in radionuclide gastrointestinal bleeding studies.[1,2] We report a patient in whom gastric bleeding was suspected on the basis of incidental findings on renal scintigraphy with Tc-99m DTPA.

A 68-year-old man was hospitalized with acute-onset chest pain and was diagnosed to have aortic dissection. Stool testing revealed occult blood. *Investigations:* hemoglobin 9.3 g/dL, white blood cell count 11,800/mm³, serum creatinine 1.12 mg/dL, blood urea nitrogen 25 mg/dL. Contrast-enhanced CT scan revealed aortic dissection extending from the descending aorta to the right iliac artery, and dysfunction of the left kidney. Renal scintigraphy with Tc-99m DTPA revealed left renal dysunction and an unexpected area of tracer accumulation in the left upper abdomen, suggesting a possibility of gastric bleeding (Fig). Gastroscopy confirmed the presence of a bleeding gastric ulcer. He was treated with H2-receptor blockers.

Tc-99m labeled RBCs and Tc-99m HAS-D are blood pool agents, and persist in the circulation for a long time, resulting in high background activity, which tends to mask the site of bleeding, sometimes with failure to

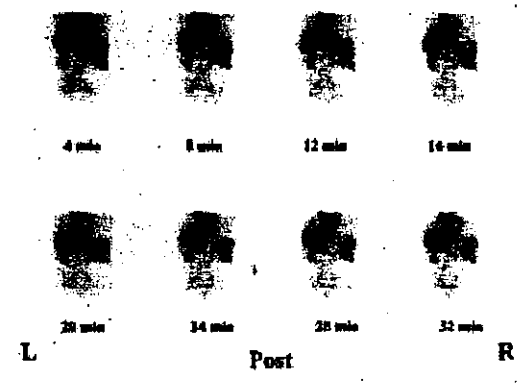

Fig: Renal scintigraphy with Tc-99m DTPA showing left renal dysfunction and tracer accumulation in left upper abdominal region, suggesting gastric bleeding