

Mitochondrial mutations in hepatocellular carcinomas and fibrolamellar carcinomas

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Mitochondrial mutations are well documented in hepatocellular carcinoma, but their role in carcinogenesis remains unclear. To clarify their significance, a comprehensive analysis was performed of hepatocellular carcinomas ($N=24$), including quantifying the total mitochondrial DNA levels, quantifying the levels of mitochondrial DNA with the common deletion, and complete sequencing of the mitochondrial control region. In addition, these studies were expanded and reinforced by analysis of fibrolamellar carcinomas ($N=15$), a unique type of liver carcinoma that has increased numbers of mitochondria on electron microscopy. Overall, approximately 50% of hepatocellular carcinomas had lower levels of total mitochondrial DNA than paired non-neoplastic tissues. Interestingly, despite their increased numbers of mitochondria, primary fibrolamellar carcinomas had lower levels of total mitochondrial DNA. In contrast, metastatic fibrolamellar carcinomas had greatly increased mitochondrial DNA levels. Overall, deletions in the control region were associated with lower total DNA levels in typical hepatocellular carcinoma, but somatic single base pair mutations were not. In fact, almost all single base pair mutations were either reversions to the wild-type sequence or known population polymorphisms, strongly suggesting they are not directly oncogenic. Complete sequencing of the entire mitochondrial genome in fibrolamellar carcinomas identified several somatic mutations, but no consistent pattern of mutations was found. Overall, the levels of the common deletion were highest in tissues with lower total mitochondrial DNA. In conclusion, control region deletions, but not somatic mutations, may influence total DNA copy numbers. Somatic control region mutations in hepatocellular carcinoma are not directly oncogenic but instead may be adaptive.

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Hepatocellular carcinomas develop because of genetic and epigenetic changes in nuclear DNA. The role for changes in mitochondrial DNA in carcinogenesis is less clear, but many studies have documented mitochondrial abnormalities in hepatocellular carcinomas. Mitochondrial DNA is more susceptible to DNA damage because of its proximity to reactive oxygen species, its lack of histones, and its more limited DNA repair capabilities. The number of mitochondria varies between cell types and each individual mitochondrion can have varying copy numbers of mitochondrial DNA, giving rise to the possibility of substantial differences in mitochondrial DNA copy numbers

between cell types and between benign and malignant tissues. Given the importance of ATP production for dividing tumor cells, mitochondrial mutations or changes in total DNA copy numbers could conceivably have important roles in the development of hepatocellular carcinoma. In fact, mitochondrial mutations and changes in total DNA copy numbers have been reported in hepatocellular carcinomas.^{1–7}

Most reported mitochondrial abnormalities in hepatocellular carcinomas can be categorized into three types. First, mutations are found in the control region,¹ a non-coding region of the mitochondrial genome that contains key regulatory elements including promoters of mitochondrial transcription. Somatic mutations in the control region include single base pair changes and deletions or insertions of a cytidine in poly C-tracts, which are located at bp positions 303–309 and 433–438.^{1–4} A second genetic lesion, the common deletion, affects the coding region of complex I of the electron transport chain. Interestingly, hepatocellular carcino-

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mas appear to select against the common deletion, as the frequency of the common deletion is often lower in carcinomas than in paired background liver tissues.^{1,2,5} Finally, total mitochondrial DNA is decreased in approximately 2/3 of hepatocellular carcinomas, range 57–77%.^{2–4,6,7} This observation is also true for other carcinomas, which show decreased total mitochondrial DNA mass and decreased oxidative phosphorylation.⁸

Despite the observations summarized above, the role for mitochondrial changes in liver cancer remains unclear. A recent analysis of mitochondrial mutations in selected cancers suggested that the changes are not oncogenic because the same somatic mutations could also be found as polymorphisms in the general population.⁹ However, hepatocellular carcinomas were not included in this study. Furthermore, animal models have shown that carcinogen-induced hepatocellular carcinomas have mitochondrial mutations.¹⁰ In addition, numerous studies of primary human hepatocellular carcinomas, as outline above, have found mitochondrial mutations. Thus, this study was designed to clarify the nature of mitochondrial changes in hepatocellular carcinomas. In addition, to further our understanding of mitochondrial changes in liver cancer, we exploited the availability of fibrolamellar carcinomas, a unique type of liver carcinoma that is known to be associated with mitochondrial changes. Fibrolamellar carcinomas have increased mitochondria by electron microscopy, which imparts a distinctive oncocytic appearance to the tumor cells on routine light microscopy.¹¹

Total mitochondrial DNA and the total proportion of mitochondria with the common deletion were quantitated in 15 fibrolamellar and 24 hepatocellular carcinomas. In addition, we sequenced the entire mitochondrial control region (bp 16 024–576) from all cases. Finally, given the known mitochondrial abnormalities in fibrolamellar carcinomas, we sequenced the entire mitochondrial genome in five primary fibrolamellar carcinomas.

Materials and methods

Tissues

Institutional Review Board approval was obtained and fresh tissues were harvested at the time of

surgery. All tissues were de-identified at the time of collection. The histological diagnoses were confirmed in all cases by routine microscopy. A total of 15 fibrolamellar carcinomas were studied, including 11 primary and 4 metastatic fibrolamellar carcinomas (Table 1). The metastases were all extrahepatic. The tumors were from 14 individuals: in one case there was both primary and metastatic tissues available for study. For 7 of the 14 individuals, paired non-neoplastic tissues were also available.

Paired tissues from 24 individuals with typical hepatocellular carcinoma were also studied (Table 1). Tissues had been collected sequentially on the basis of the availability and viability of tumor and were included in this study on the basis of the adequacy of tissue. These cases were split between carcinomas that arose in cirrhotic livers ($N=12$) and those that arose in non-cirrhotic livers ($N=12$). Non-cirrhotic cases serve as an important control for fibrolamellar carcinomas, which arise in non-cirrhotic livers. All of the tumors in cirrhotic livers had chronic viral hepatitis as an underlying cause of liver disease (Table 1).

Quantification of Total Mitochondrial DNA and Common Deletion

All tissues were examined for total wild-type mitochondrial DNA levels and for the total levels of mitochondrial DNA with the common deletion. For those fibrolamellar carcinomas that did not have paired non-neoplastic tissues, including the metastases, the average value of all the non-neoplastic tissues with fibrolamellar carcinoma was used as the non-neoplastic comparator.

The QIAmp DNA mini kit (Qiagen, Valencia, CA, USA) was used to extract DNA from 20 to 25 mg of tissue. Mitochondrial DNA was quantified by real-time PCR using previously reported primers¹² and normalized to nuclear DNA using external standard controls. The external controls were created using cloned mitochondrial DNA fragments with and without the common deletion and absolute values calculated. Because the amount of total nuclear DNA in a tumor can be affected by the degree of aneuploidy, a region of nuclear DNA that is typically not involved by chromosomal gains and losses in

Table 1 Demographical findings

Tumor type	Cases (N)	Age average years \pm s.d.	Gender, M/F	Ethnicity W/B/A
Fibrolamellar carcinomas, primary	11 ^a	26 \pm 12	4:7	11:0:0
Fibrolamellar carcinomas, metastatic	4 ^b	24 \pm 8	2:2	4:0:0
Hepatocellular carcinomas in non-cirrhotic livers ^c	12	55 \pm 20	7:5	10:1:1
Hepatocellular carcinomas in cirrhotic livers ^d	12	58 \pm 11	9:3	9:2:1

^aOf these 11 cases, 7 had paired non-neoplastic liver tissues.

^bOne of these individuals also had a primary tumor available.

^cThese hepatocellular carcinomas arose in non-cirrhotic livers with no known underlying cause of liver disease.

^dAll of the hepatocellular carcinomas that arose in cirrhotic livers had hepatitis C ($N=8$) or hepatitis B ($N=4$) as their underlying cause of liver disease.

fibrolamellar and hepatocellular carcinomas was chosen for normalization purposes. The Progenetix website (<http://www.progenetix.net/progenetix>) contains numerous dendrograms showing chromosomal gains and losses in liver cancer, and a region of the nuclear genome that was relatively free from dysploidy (chromosome 2q24) was chosen, as well as real-time PCR primers designed to amplify the ABCB11 gene within this region (primers available on request). For comparing the common deletion frequency among cases, a common deletion index was created as the ratio of mitochondrial DNA with the common deletion to the total mitochondrial DNA, $\times 10^4$.

Sequencing of Mitochondrial Control Regions

The entire control region was amplified (primers available on request) and directly sequenced from all 24 typical hepatocellular carcinoma and all 15 fibrolamellar carcinomas, as well as paired non-neoplastic tissues. Sequences were compared with the Revised Cambridge Reference Sequence for mitochondrial DNA (GeneBank REFSEQ: AC_000021.2). Somatic mutations were identified for all cases with paired non-neoplastic tissues.

Sequencing of Full-Length Mitochondrial DNA

The full-length mitochondrial DNA was examined in paired non-tumor and tumor tissues from five primary fibrolamellar carcinomas with sufficient tissues and one typical hepatocellular carcinoma control. In addition, all four metastases were fully sequenced. The mitochondrial DNA was amplified using previously reported primers¹³ as well as additional supplemental primers (available on request) and directly sequenced. For analysis, sequences were compared with the Revised Cambridge Reference Sequence.

Evaluation of OXPHOS Proteins

Western blots were performed to evaluate the protein production of nuclear-encoded and mitochondrial-encoded proteins. A cocktail containing five monoclonal antibodies to various OXPHOS proteins (Mitosciences, Eugene, OR, USA) was used to simultaneously examine the levels of five different mitochondrial proteins. Four are nuclear-encoded proteins: complex I subunit, *NDFUB8* (protein, gene symbol); complex II subunit, *SDHB*; complex III subunit Core 2, *UQCRC2*; and complex V or ATP synthase subunit- α , *ATP5A1*. One is a mitochondrial-encoded protein: complex IV subunit II, *COXII*. Human heart mitochondrial proteins were used as a positive control.

Fifty to 75 mg of liver tissue were homogenized in phosphate-buffered saline containing a protease inhibitor cocktail (Roche). Total protein was quantitated using the BCA protein assay (Pierce). Protein concen-

trations were adjusted to 5 mg/ml and a detergent (Mitosciences) was added and samples incubated on ice for 30 min. The supernatant was then stored at -80°C until further analysis. A total of 70 μg of protein was loaded for each lane on the western blots.

Results

Quantitation of Mitochondrial DNA Levels

In cases of fibrolamellar carcinomas, the background liver tissues showed decreased total numbers of mitochondrial DNA when compared with the non-cirrhotic liver tissues (386 ± 166 vs 1480 ± 1570 total mitochondrial genomes per ABCB11 gene; data shown as mean \pm s.d., Student's *t*-test $P=0.03$). However, the non-neoplastic livers did not differ between those with fibrolamellar carcinoma and those with cirrhosis (386 ± 166 vs 789 ± 739 ; Student's *t*-test $P=0.09$) (Figure 1a).

Fibrolamellar carcinomas had less total mitochondrial DNA than hepatocellular carcinomas that arose in non-cirrhotic livers (534 ± 395 vs 1072 ± 733 ; Student's *t*-test $P=0.04$). The differences between tumors in cirrhotic livers and fibrolamellar carcinomas were not significant (534 ± 395 vs 1337 ± 2286 ; Student's *t*-test $P=0.27$) (Figure 1b). In contrast, metastatic fibrolamellar carcinomas had more mitochondrial DNA (4339 ± 2001) than primary fibrolamellar carcinomas as well as both groups of hepatocellular carcinomas (all Student's *t*-tests $P<0.05$) (Figure 1b). Overall, the mitochondrial DNA levels did not correlate between the tumors and their paired non-neoplastic tissues (Pearson $R^2=0.04$).

Common Deletion Index

The common deletion index was higher in the non-neoplastic livers of cases with fibrolamellar carcinomas (1.9 ± 2.7 ; mean \pm s.d.) than in the non-cirrhotic (1.3 ± 1.3) and cirrhotic livers (1.1 ± 1.5), although the difference was of borderline statistical significance (Figure 1c; Student's *t*-tests, $P=0.05$ and 0.06 , respectively). Within the tumors, there was retention of mitochondrial DNA containing the common deletion in fibrolamellar carcinomas (5.9 ± 7.9), but not in the typical hepatocellular carcinomas that arose in non-cirrhotic (0.1 ± 0.3 ; Student's *t*-test $P=0.045$ compared with fibrolamellar carcinoma) and cirrhotic livers (0.3 ± 0.3 ; Student's *t*-test $P=0.040$ compared with fibrolamellar carcinoma). Interestingly, the common deletion index for metastatic fibrolamellar carcinomas (0.1 ± 0.001) reverted to levels similar to typical hepatocellular carcinoma (Figure 1d).

Mitochondrial Control Region Base Pair Changes

The control region is 1123 bp in length, extending from nucleotide positions 16 024 to 576. It is

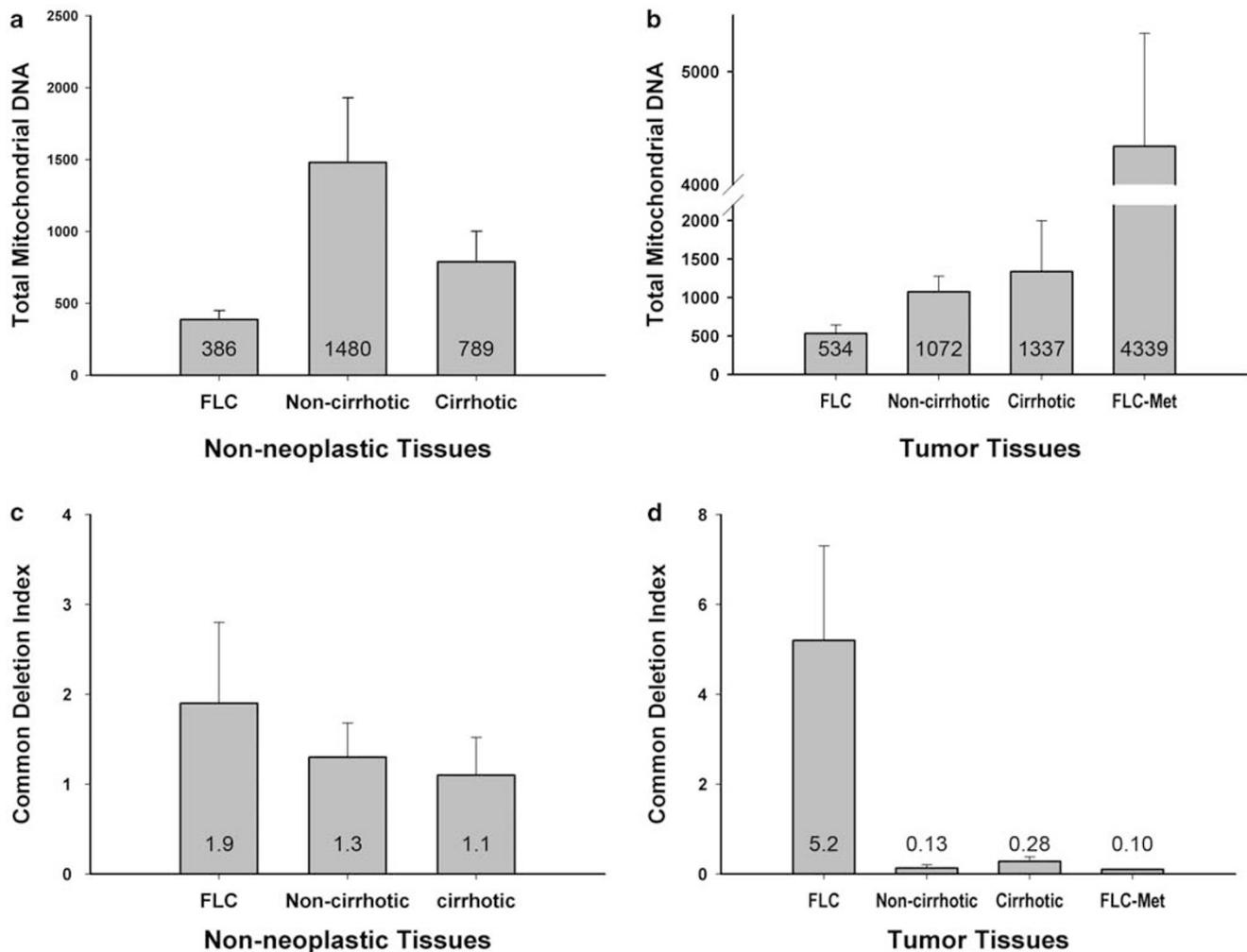


Figure 1 In all panels, the group mean is shown as well as the standard errors of the means. Total mitochondrial DNA levels are lower in the background liver tissues of individuals with fibrolamellar carcinoma and cirrhosis (a). In tumor tissues, primary fibrolamellar carcinomas have the least amount of total mitochondrial DNA, while levels did not differ between hepatocellular carcinomas arising in cirrhotic versus non-cirrhotic livers (b). Interestingly, metastatic fibrolamellar carcinomas had very high levels of total mitochondrial DNA (b). The common deletion index did not differ in non-neoplastic tissues (c). However, in tumor tissues, fibrolamellar carcinomas showed retention of mitochondrial genomes with the common deletion, while hepatocellular carcinomas and metastatic fibrolamellar carcinomas did not (d).

composed of two hypervariable regions on the ends, at positions 16 024–16 383 and 57–372, with a central conserved region. In comparison with the Cambridge Reference Sequence, a total of 100 (9%) of the 1123 nucleotides of the control region were targets for variation within the non-neoplastic or neoplastic liver tissues, excluding poly-C tract changes and deletions that are discussed further below. When there was variation at any given site, in nearly all cases the variation was limited to a single-specific change. For example, at position 16 223 a total of 19 samples differed from the reference C. However in all 19 cases the change was to a T. In only 5/100 positions was more than one specific nucleotide substitution identified. The most common changes were T to C ($N=34$ locations), C to T (31), A to G (16), and G to A (12), with a transition to transversion ratio of 7:1. However, the nucleotide positions that were subject to substitutions were not

uniformly distributed and there was a strongly conserved region of approximately 330 bp that extended from 16 393 to 54 (Figure 2a) overlapping very closely with the known conserved region from 16 384 to 56. As expected, this region was bracketed by two hypervariable regions. These observations indicate that the mitochondrial DNA changes, when defined by differences from the reference sequence, largely represent polymorphisms within the hypervariable regions.

To further confirm this, we analyzed the mutation/polymorphism data reported on Mitomap,¹⁴ which shows reported base pair changes in a wide range of benign and malignant tissues for the hypervariable region. The frequency of mutations at each base pair position was plotted and clearly revealed the same basic pattern that we found in liver tissues: most reported changes cluster in the two hypervariable regions (Figure 2b), leaving a central strongly conserved region.

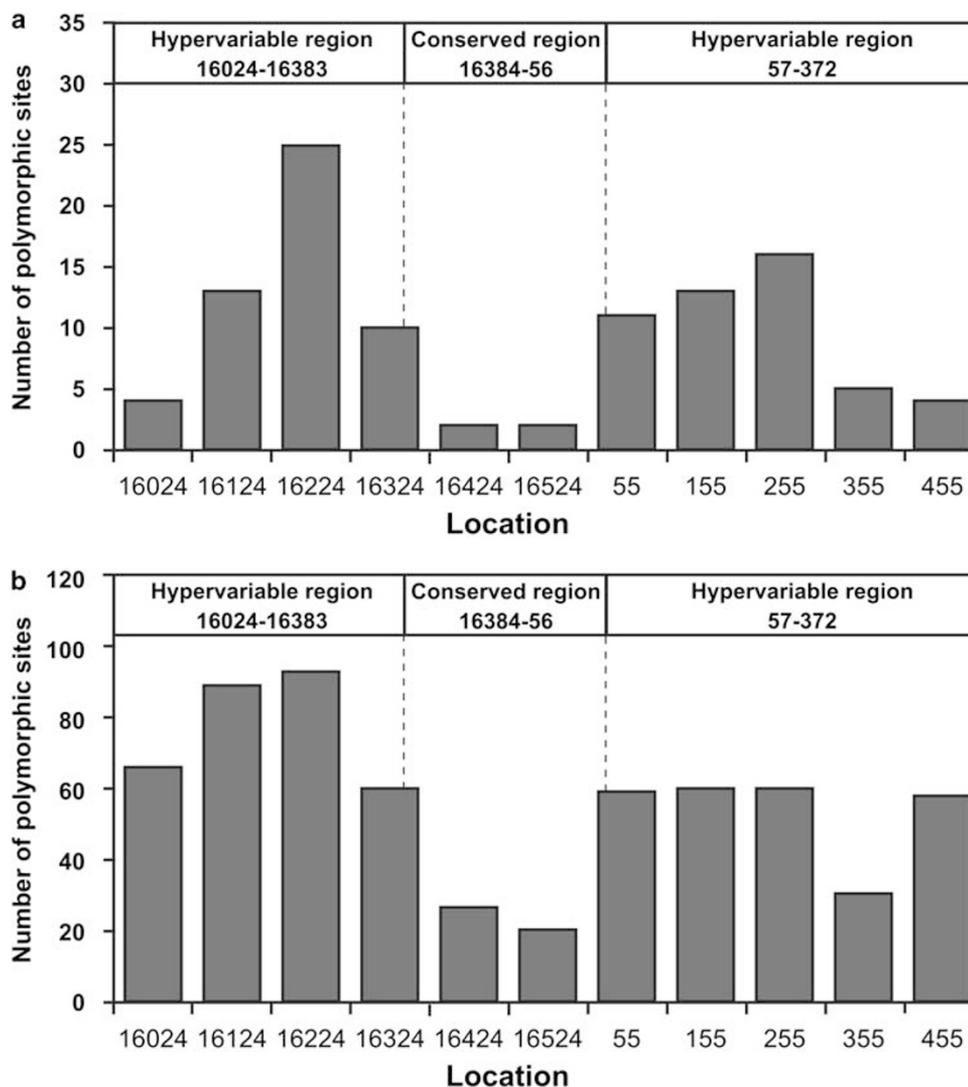


Figure 2 Within the control region, benign or malignant liver tissues had sequence differences from the reference sequence that affected 100 of 1123 positions within the control region. These positions were located at the two ends, with a central conserved region (a). The same pattern was found with analysis of data from Mitomap, which includes data from a variety of benign and malignant tissues (b).

A total of 17 somatic mutations composed of single base pair substitutions were found within the control region in 12 carcinomas: 4/7 fibrolamellar carcinomas, 3/12 non-cirrhotic hepatocellular carcinomas, and 5/12 cirrhotic hepatocellular carcinomas (Table 2). All of the mutations were located within the hypervariable regions. Interestingly, in 16/17 instances the somatic mutations represented either reversion from a polymorphism in the background liver tissue to the nucleotide found in the reference sequence ($N=8$) or can be found as a polymorphism in the general population ($N=8$), as reported in MitoMap.¹⁴ Repeat amplification and sequencing ensured that the somatic mutations do not represent amplification or sequencing artifacts. These observations indicate that most somatic mutations in the mitochondrial control region are not unique to carcinoma even if they are tumor-specific for any given case.

The control region contains two poly-C tracts that have been previously identified as having mutations in hepatocellular carcinomas. The first poly-C tract is located at bp 16 184–16 194, and somatic mutations were found within four hepatocellular carcinomas (Table 3). Interestingly, in each case the wild-type sequence in the non-neoplastic tissues lacked the middle T, suggesting a T to C change for this central T predisposes to subsequent somatic mutations in this poly-C tract. The second poly-C tract is located at bp 303–315, and somatic mutations were identified in seven cases, six of which were C insertions or deletions within the first portion of the poly-C tract (Table 3). A single case had an additional mutation located in a third poly-C tract (Table 3). Overall, 13 (42%) carcinomas had somatic mutations in at least one of the poly-C tracts. A single case had somatic mutations in both the 16 184–16 194 and 303–315 poly-C tracts.

Table 2 Somatic mutations within the control region

Base pair position	Change	Group	Notes
16 093	Y to T	Fibrolamellar carcinoma	Reversion ^a
16 124	T to Y	Hepatocellular carcinoma, cirrhotic	Polymorphism ^b
16 263	C to T	Hepatocellular carcinoma, cirrhotic	Reversion
16 337	C to Y	Hepatocellular carcinoma, cirrhotic	Polymorphism
16 390	G to A	Hepatocellular carcinoma, non-cirrhotic	Polymorphism
	G to A	Fibrolamellar carcinoma	Polymorphism
60	T to C	Fibrolamellar carcinoma	Polymorphism
	T to C	Hepatocellular carcinoma, non-cirrhotic	Polymorphism
64	Y to T	Fibrolamellar carcinoma	Polymorphism
72	T to C	Hepatocellular carcinoma, cirrhotic	Polymorphism
	C to T	Hepatocellular carcinoma, cirrhotic	Reversion
146	C to T	Hepatocellular carcinoma, non-cirrhotic	Reversion
186	C to T	Fibrolamellar carcinoma	Polymorphism
189	A to G	Fibrolamellar carcinoma	Polymorphism
366	G to R	Fibrolamellar carcinoma	
442	Y to T	Hepatocellular carcinoma, non-cirrhotic	Reversion
477	C to T	Hepatocellular carcinoma, non-cirrhotic	Reversion

Some carcinomas had more than one mutation.

^aThe somatic mutation in the tumor changed a polymorphism in the non-neoplastic liver back to the wild-type sequence reported for the Cambridge Reference sequence.

^bReported in MitoMap as a polymorphism.

Table 3 Poly-C tract somatic mutations within the control region

Type of change	Location	Base pair, change	Fibrolamellar carcinoma	HCC, non-cirrhotic	HCC, cirrhotic
Singe base pair	16 184–16 194 ^a	C insertion or deletion	0	3	1
Singe base pair	303–315 ^b	C insertion or deletion	1	3	3
Singe base pair	303–315 ^c	306, C to M	0	1	0
Singe base pair	979–984 ^c	C insertion	0	1	0
Larger poly-C tract deletion	312:321 ^d	Deletion	2	0	0
Larger poly-C tract deletions	306:357 ^d	Deletion	1	0	0
Larger poly-C tract deletions	311:321	Deletion	0	1	0
	311:320	Deletion	0	1	0
	16 480:16 5	Deletion	0	1	0
	22	CA deletion	0	1	0
	522 ^e				
Larger poly-C tract deletions	16 491:16 500	Deletion	0	0	1

^aWild type is C₅TC₄.

^bWild type is C₇TC₅.

^cWild type is C₆.

^dThese two deletions are in tumors from the same individual. The deletion is larger in the metastasis than in the primary tumor but targets the same area.

^eReported polymorphism in MitoMap.

Control region deletions were found in eight tumors (Table 3). Deletions were not present in non-neoplastic tissues. Interestingly, in five of the eight cases, the deletion at least partially involved the 303–315 poly-C tract. In one case of fibrolamellar carcinoma, the tumor had a somatic deletion within the 303–315 poly-C tract that became significantly larger in the metastases (Table 3).

Overall, tumor mitochondrial DNA levels were lower than paired non-neoplastic tissues in 5/11 fibrolamellar and 11/24 typical hepatocellular carcinomas. No association was found between mitochondrial DNA levels and somatic control region mutations composed of single base pair substitutions

($P=0.47$) or poly-C tract mutations ($P=0.42$). There was a borderline-significant trend toward lower mitochondrial DNA levels in cases of hepatocellular carcinoma with deletions ($P=0.058$). Further analysis of cases with any type of mitochondrial control region mutation vs those without any type of mutations found no association with DNA levels ($P=0.46$).

Full-Length Mitochondrial Sequences

Sequencing of the full-length mitochondrial genome in fibrolamellar carcinomas showed a total of eight somatic mutations outside of the control region

(Table 4). Three tumors had two somatic mutations each and the remaining two tumors had one mutation each. One of the mutations is predicted to lead to an amino-acid change, whereas three mutations are synonymous, and the remaining four involve either the 16S rRNA or tRNA. Two fibrolamellar carcinomas had T to C transitions targeting the same base pair of ND1, but no amino-acid change is predicted. A single primary fibrolamellar carcinoma had a somatic deletion of bp 15 240–15 247, located within ND6. No somatic mutations or deletions were found in the mitochondrial DNA of the single typical hepatocellular carcinoma that was completely sequenced.

In one case, tissues from the same individual were available from non-neoplastic liver, primary tumor, and metastatic tumor (Table 5). The metastatic tumor had two mutations not present in either the non-neoplastic liver or the primary tumor, both of which targeted the 16S rRNA. One mutation has been reported as a polymorphism in Mitomap, whereas the other has not. An additional somatic mutation was present in both the primary and metastatic tumor, but represents a reversion from a polymorphism in the non-neoplastic tissues back to the nucleic acid present in the Cambridge Reference Sequence (Table 5).

Table 4 Mitochondrial sequences from primary fibrolamellar carcinomas were compared with the paired non-neoplastic liver tissues

Base pair location, change	Gene	Amino-acid change	Notes
1916, G to C	16S rRNA	Non-coding	
4248, T to Y ^a	ND1	Synonymous	
4248, T to C ^b	ND1	Synonymous	
4824, A to R ^a	ND2	T to A	Polymorphism
5650, G to A ^c	A	Non-coding	
6797, C to T ^c	COI	Synonymous	
10463, C to T	R	Non-coding	Reversion
15975, G to A ^b	P	Non-coding	

Somatic mutations within the region of bp 475–15 985 are shown. ^{a,b,c}Indicates same tumor. No somatic mutations were found in the control hepatocellular carcinomas and paired non-neoplastic liver that was entirely sequenced.

Table 5 Fibrolamellar case number 5 had normal liver, primary tumor, and metastatic tissues available, and the whole mitochondrial genome of each was analyzed

Base pair location	Gene	AA change	Reference sequence	Non-neoplastic liver	Primary tumor	Metastasis
2300	16S rRNA	Non-coding	G	G	G	A ^a
3118	16S rRNA	Non-coding	T	T	T	C
10 463	R	Non-coding	T	C	T	T ^b

Sequence changes are shown. The reference sequence data are also shown.

^aReported polymorphism in MitoMap.

^bReversion in tumor and metastatic deposit back to reference sequence.

Analysis of OXPHOS Proteins

Western blots of paired tumors and non-tumor tissues showed decreased OXPHOS protein levels in seven hepatocellular carcinomas that arose in cirrhotic livers and eight hepatocellular carcinomas that arose in non-cirrhotic livers (Figure 3). Interestingly, however, the fibrolamellar carcinomas showed decreased nuclear-encoded and mitochondrial-encoded OXPHOS proteins in only one case. Overall, no correlation was evident between the western blot findings and the total mitochondrial DNA levels within the tumors.

Discussion

This study provides a comprehensive analysis of mitochondrial changes in hepatocellular carcinomas, including a simultaneous examination of

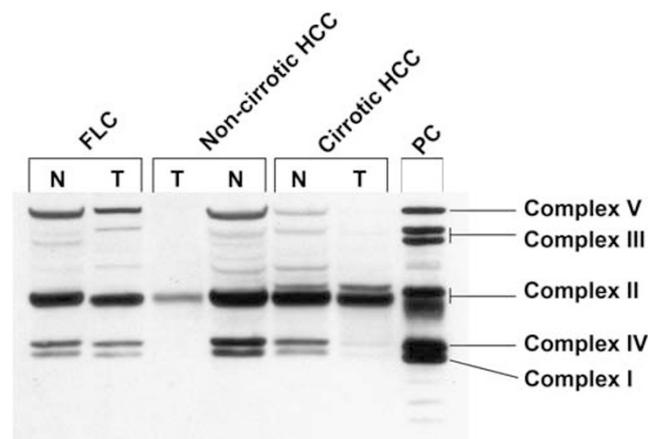


Figure 3 Western blots show decreased levels of nuclear-encoded mitochondrial proteins in typical hepatocellular carcinomas (HCC) but not fibrolamellar carcinomas (FLC). A cocktail containing five monoclonal antibodies to various OXPHOS proteins was used to simultaneously examine the levels of five different mitochondrial proteins. Four are nuclear-encoded proteins: complex I, complex II, complex III, and complex V. One is a mitochondrial-encoded protein: complex IV. Protein levels appear decreased in hepatocellular carcinomas compared with paired non-neoplastic tissues. In contrast, fibrolamellar carcinoma did not show decreased protein levels. Human heart mitochondrial protein was used as a positive control (PC). For complexes II and III, two bands each can be seen, which represent either isoforms or degradation.

control region mutations, total mitochondrial copy numbers, and mitochondrial common deletion copy numbers. These results significantly clarify how these mitochondrial changes are inter-related and how they are linked to hepatocellular carcinoma.

Total mitochondrial DNA levels were found to be different in hepatocellular carcinomas compared with paired non-neoplastic tissues, but the difference depends on the background liver: in non-cirrhotic livers, hepatocellular carcinomas tend to have less mitochondrial DNA, whereas in cirrhotic livers the hepatocellular carcinomas tend to have more mitochondrial DNA. Others have also reported that mitochondrial DNA copy numbers in hepatocellular carcinoma correlate with cirrhosis.⁶

Control region deletions in this study were associated with lower total mitochondrial DNA levels. Control region deletions heavily clustered into an area that at least partially involved the poly-C tract at bp positions 303–309. Because these deletions are at a low frequency, they have received relatively little attention, but it is of interest to note that at least five other studies have also reported control region deletions that targeted this same area.^{3,4,15,16} These deletions are attractive candidates for having an oncogenic potential, given their recurrent presence in hepatocellular carcinomas and given that they have not been reported as polymorphisms.

In contrast, somatic mutations were not associated with mitochondrial DNA levels. In comparison with the Cambridge Reference Sequence, base pair changes in the mitochondrial control region are common within both non-neoplastic livers and hepatocellular carcinomas. However, nearly all of these changes are polymorphisms located within the hypervariable portions of the control region. Furthermore, nearly all control region somatic mutations (17/18) can be found as polymorphisms in the general population or represent reversions back to the Cambridge Reference Sequence. In fact, a re-examination of data from hepatocellular carcinomas reported by others confirms this key point: 6/6 of the somatic single base pair mutations in the control region found by Okochi *et al*¹⁵ are also listed as polymorphisms on Mitomap, whereas 12/13 mutations found by Wong *et al*⁴ and 12/14 mutations reported by Lee *et al*⁷ represent either polymorphisms or reversion back to the reference sequence. Together, these findings strongly support a model in which control region mutations in hepatocellular carcinoma are not oncogenic but instead may be adaptive. Brandon *et al*⁹ came to a similar conclusion after analysis of a database of mitochondrial mutations that included ovarian, head and neck, bladder, thyroid, and prostate cancers. In their study, they found 72% of somatic mutations outside the control region, and 85% of somatic mutations within the coding region could also be found as sequence variants within the general population. The data from our study provides direct and

independent evidence that supports this overall model for control region somatic mutations, and, of note, is from hepatocellular carcinomas, a type of carcinoma not included in their database analysis.

Others have also argued, on the basis of computer modeling, that homoplasmic mitochondrial mutations in tumors can be explained by random processes and that selection is not necessary.¹⁷ In this model, the probability of containing homoplasmic mutations is based on mitochondrial mutation frequencies, the number of cell divisions, and random processes, but selection on the basis of adaptive fitness is not required. Our results do not exclude this possibility, but clearly show that the same processes (whether it be adaptive selection or random drift) are active in both human populations and in tumors. Furthermore, the observation that they lead to remarkably similar results and that the types of somatic mutations in tumors are strongly constrained suggest a role for adaptive selection.

In terms of the common deletion, the findings in this study indicate a strong relationship to the overall mitochondrial copy number: when total mitochondrial DNA numbers are low, there is a relatively greater retention of the 4977 bp deletion. This important observation is evident in our study where the background livers of patients with fibrolamellar carcinoma have fewer total mitochondria and greater numbers of mitochondria with the 4977 bp deletion. Others have also reported that non-neoplastic liver tissues from males, in comparison with females, have lower total mitochondrial DNA levels but higher levels of the common deletion.² Likewise, in alcoholic patients, liver tissues have fewer total mitochondria and higher numbers of the common deletion than controls.²

The energy requirements of tumors are often met by aerobic glycolysis, a phenomenon termed the Warburg effect, where tumors actively consume oxygen through OXPHOS yet at the same time produce abundant lactic acid when metabolizing glucose. This process is associated with decreased mitochondrial numbers and decreased OXPHOS protein levels and appears to be regulated by the overexpression of Hexokinase II, an enzyme that allows tumor cells to aberrantly harness OXPHOS proteins to drive glycolysis for ATP production under low oxygen conditions.¹⁸ Decreased OXPHOS proteins in hepatocellular carcinoma were identified in this and other studies.² It is unclear from the available data how the mitochondrial abnormalities evident in hepatocellular carcinomas, both in copy numbers and the somatic mutations, are linked to hexokinase II expression. However, it is of interest to note that the metastatic fibrolamellar carcinomas, which presumably are no longer under low oxygen conditions and would thus be less dependent on hexokinase II expression, have a dramatic increase in their mitochondrial DNA copy numbers.

Fibrolamellar carcinomas have morphologically evident mitochondrial abnormalities that are not

seen in most typical hepatocellular carcinomas. Despite this, fibrolamellar carcinomas were not enriched for control region mutations. Furthermore, sequencing of the entire mitochondrial genome did not identify consistent or frequent DNA changes. Indeed, as with somatic mutations in the control region, most of the somatic mutations outside the control region were found to represent either reversion to the reference sequence or polymorphisms. Taken together, these findings suggest that primary mitochondrial DNA changes do not explain the increased mitochondrial mass in fibrolamellar carcinomas and suggest a role for defects in mitochondrial biogenesis.

These data do not imply that mitochondrial mutations are not biologically relevant. There is substantial data that mutations in nuclear-encoded proteins can be oncogenic and it seems likely that the same metabolic pathways could be disrupted, and oncogenic, by mutations in mitochondrial-encoded proteins. Their identification, however, is made more difficult because of the frequency of adaptive/random somatic mutations. The role for adaptive mutations, as well as for mutations involving non-protein encoding genes such as the 16sRNA, requires further exploration.

In conclusion, control region deletions are associated with lower mitochondrial DNA levels and their recurrent presence in hepatocellular carcinoma suggests a potential oncogenic role. In contrast, somatic mutations in the coding region do not adequately explain the variation in tumor mitochondrial DNA numbers. Furthermore, most control region single base pair mutations are not oncogenic because they are also present as polymorphisms in the general population. These observations are expanded and reinforced by analysis of fibrolamellar carcinomas, which do not have consistent patterns of mitochondrial DNA changes despite their mitochondrial abnormalities.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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