Quantification of mitochondrial sublimons in human fibrillating atria

Nidal MAAROUF*, Gavin ARNO*, Nicholas D. CARTER†, Petros SYRRIS†, Shamil YUSUF*, A. John CAMM*, Jan POLEINIKI and Naab M. Al-SAADY*

*Department of Cardiovascular Medicine, St George’s Hospital Medical School, Cranmer Terrace, London SW17 0RE, U.K., and †Department of Clinical Developmental Sciences, St George’s Hospital Medical School, Cranmer Terrace, London SW17 0RE, U.K.

ABSTRACT

Supraventricular tachycardias, including AF (atrial fibrillation), and mtDNA (mitochondrial DNA) deletions may lead to dilated cardiomyopathy. It is unknown whether mtDNA function is impaired in the human atrium in AF. In the present study, we investigated the role of rearranged mtDNA ‘sublimons’ in the pathogenesis of AF. Right atrial biopsies were collected from 38 patients in AF and 35 patients with SR (sinus rhythm) undergoing elective cardiac surgery. Total DNA was extracted by standard methods. The break-point regions of the two most prevalent classes of sublimon were amplified by PCR using fluorescent oligonucleotides for the 3.75 kb partial duplication and the 2.83 kb deletion. Multiplex reactions included additional primers to amplify an internal genomic standard for semi-quantitative analysis. Reaction products were quantified as peak areas in the electrophoretogram and ratios computed of the sublimon abundance relative to the genomic standard. There was no difference in SCN (sublimon copy number) between AF and SR patients [19.09 ± 28.29 compared with 10.25 ± 24.68, the difference was 0.28 (95% confidence interval, −0.04 and +0.61; P = 0.08)]. SCN did not increase with age (P = 0.207) and was unrelated to AF duration (P = 0.661), left atrial diameter (P = 0.560), post-operative AF (P = 0.52), underlying disease (P = 0.94), medication and gender (2.84 ± 0.72 in females vs 2.97 ± 0.67 in males; P = 0.431). In conclusion, our findings do not indicate any role of mtDNA in the pathophysiology of AF.

INTRODUCTION

Virtually all tissues in the body depend to some extent, and cardiac tissue almost exclusively, on mitochondria for energy production by producing ATP through oxidative phosphorylation. Each cardiac cell contains 2000–3000 mitochondria and each mitochondrion possesses two or three mtDNA (mitochondrial DNA) genomes. Deficiency of this system can cause cardiac abnormalities [1].

mtDNA deletion in skeletal myocytes is associated with skeletal muscle myopathy; however, an aetiological relationship between myopathy and cardiomyopathy has been suspected, because some patients with skeletal myopathy also show cardiomyopathic changes. Furthermore, it was found that abnormally accelerated mtDNA mutations, especially those related to mitochondrial protein synthesis, can induce premature aging and severe mitochondrial cardiomyopathy [2]. Li et al. [3] found that mtDNA damage accumulates in the myocardium.
of patients with dilated cardiomyopathy. Persistent supraventricular tachycardias, including AF (atrial fibrillation), have been shown to induce myopathy (tachycardiomypathy), both in animal models [4] as well as in humans [5]. Although evidence from animal models of AF showed mitochondrial structural changes [6], it is still unclear whether tachycardiomypathy is a cause or consequence of mitochondrial changes. However, in these animal models as well as in man, AF is associated with atrial dilation and structural changes [7,8]. Furthermore, these changes correlate with the duration of AF [9,10].

In a study of human cardiac tissue [11], it was found that a series of adjacent slices of the atrial myocardium contained a vastly different spectrum of multiple mtDNA deletions. Kajander et al. [12] described low abundance rearranged mtDNAs and named them ‘sublimons’. The same authors [13] have studied these sublimons in several organs, including the heart, concluding that human mtDNA sublimons represent a pool of variant molecules that can become amplified under pathological conditions and contribute to cellular dysfunction. They stated that sublimons are tissue-specific and the prevalent sublimons in the heart were 3.75 kb and 2.83 kb classes. Nevertheless, no correlation was found between sublimon abundance and any common cardiac disease phenotype when 300 post-mortem ventricular autopsies were studied [14]. The latter study analysed sublimons in conditions such as ischaemic heart disease, cardiomyopathies and lethal arrhythmias, but no AF patients were studied.

To our knowledge, no published studies have addressed the potential link between mtDNA deletion and AF. However, there is a small study by Tsuibo et al. [15] of mtDNA deletions in 26 human right atria samples showing an association between mtDNA deletion and age. Only seven of these patients were in AF and all had mtDNA deletions. These workers also suggested that mtDNA deletion was associated with ATP deficiency. Therefore the aim of the present study was to determine if there was a specific link between AF and mtDNA sublimons. In particular, we tested the hypothesis that there is a relationship between mtDNA sublimons and age, LAD (left atrial diameter), duration of AF and POAF (post-operative AF).

**METHODS**

**Patients and sample collection**

Tissue from the RAA (right atrial appendage) was obtained from patients undergoing CABG (coronary artery bypass grafting), MVR or AVR (mitral or aortic valve replacement/repair) surgery from two groups of patients with no known overt mitochondrial disease. Group 1 had permanent AF, and group 2 were sex-matched controls in sinus rhythm (SR). Patients gave written informed consent and were undergoing elective cardiac surgery in the Cardiothoracic Unit at St. George’s Hospital, London. Clinical details, including age, sex, type of surgery, duration of AF, LAD and POAF in the SR group were recorded for each patient. Seventy-six patients were considered eligible for this study, 40 of whom were in AF and 36 were in SR (for complete details of the study patients, see supplementary data at http://www.clinsci.org/cs/106/cs1060653add.htm).

**Sample preparation**

All RAA samples were immediately cut into multiple slices depending on the initial size of the tissue, except in 14 patients where only one slice per sample was obtained. Slices were then snap-frozen in liquid nitrogen and stored at −80 °C for later evaluation.

**mtDNA extraction**

Total cellular DNA was extracted using the QIAamp DNA mini-kit from Qiagen, according to the manufacturer’s protocol.

**Fluorescent PCR**

Fluorescent PCR to characterize specific sublimon classes was performed as described previously [13]. Briefly, fluorescent primer mt16153FAM (where FAM is carbooxyfluorescein-5-succinimidyl ester) was used in addition to either primer mt3150 or mt2204 [13]. Multiplex reactions also included primers DralR and DralFROX as an internal genomic standard to perform semi-quantitative analysis. Following denaturation at 95 °C for 3 min, a PCR cycle consisting of 30 s at 95 °C, 20 s at 59 °C and 20 s at 72 °C was repeated 30 times, and the fluorescent products were analysed by capillary electrophoresis using GeneScan software on an Applied Biosystems 310 Genetic Analyser.

To verify the method, samples were tested at 1/5 and 1/25 dilutions and the ratios of the sublimon to the genomic standard were compared between different dilutions (Figure 1). Frequently, 1/25 diluted samples yielded results under the detection threshold. In such cases, the results from the 1/25 dilution were discarded. Unaltered ratios at undiluted and 1/5 dilution suggest that the data obtained represent true values of sublimon abundance and confirm the previously published method [13].

The mitochondria-negative cell line, A459 lung carcinoma-derived \( \rho^- \) cells, was used as a negative control for the assay (kindly provided by Dr I. Holt, MRC Dunn Human Nutrition Unit, Cambridge, U.K.).

**Analysis of PCR products**

Reaction products resolved at the nucleotide level were quantified as peak areas in the electrophoretogram. Ratios of the amount of sublimon product versus the genomic standard were calculated to extrapolate a mean SCN
Figure 1 Semi-quantitative multiplex fluorescent PCR analysis of the PCS (3.75 kb) without dilution (A), after 1/5 dilution (B) and after 1/25 dilution (C)

(sublimon copy number)/cell, i.e. twice the number of genomic equivalents, for all sublimons of the prevalent ‘3.75 kb class’ detected by the primers.

Statistical analysis
For comparison between groups, an unpaired Mann–Whitney U test was used. Correlation and regression were carried out for PCS (prevalent class sublimon) count against age, LAD, AF duration (in the AF group) and underlying disease. Analysis of SCN was done after log-transformation in order to make the distribution more nearly normal. We refer to this in the text as ‘log SCN’. Student’s t test for two samples assuming unequal variances was used in the SR group to observe the difference in PCS between patients who developed POAF and those who did not. Univariate and multivariate analyses were used to compare the medication effect in the SR group and POAF subgroup. Values are expressed as means ± S.D. A P value < 0.05 was considered to be statistically significant. The age range was divided according to tertiles.

RESULTS

The presence and abundance of the most prevalent class of sublimons (3.75 kb partial duplications), alongside a genomic DNA standard for quantification and validation of the assay, were investigated in the DNA samples obtained from the RAA of 76 patients undergoing elective cardiac surgery. Three patients were excluded from the study, two in AF group and one in SR group, since the genomic control was below the detection limit. Of the remaining 73 patients, 38 were in the AF group and 35 in the SR group.

PCSs were detected in 60 patients (82.19 %), 33 in AF (86.84 %) and 27 (77.14 %) in SR patients (for an example of the PCR analysis, see Figure 2). The remainder were below the detection limit (approx. < 0.1 copies per cell). The mean SCN ranged variably up to 142.084 copies/cell (mean ± S.D., 14.85 ± 26.81). Very similar SCN values were obtained from different samples for the same patient, where available.

The 2.83 kb sublimon class was below the detection limit in all SR patients (100 %) and in 34 out of 38 (90 %) AF patients. The mean SCN/cell for this type of sublimon was (1.35 ± 1.64; n = 6). Therefore this class of sublimons was excluded from analysis, since it comprised a negligible number.

mtDNA sublimon in AF and SR groups
SCN/cell in the AF group was 19.09 ± 28.29 compared with 10.25 ± 24.68 in the SR group. The difference in log SCN was 0.28 (95 % confidence interval, − 0.04 to + 0.61; P = 0.08, R² = 0.04).

mtDNA sublimon 3.75 and age
The mean age was 66.42 ± 9.08 (n = 73) years for all patients. In patients with undetectable PCS, the mean age was 66 ± 9.64 years (n = 13). The log SCN was plotted against age in the 73 patients and no statistically significant relationship between age and log SCN was found (P = 0.207, R² = 0.02; Figure 3). Mean ages for three groups defined by the 33.3rd and 66.6th percentiles of sublimon abundance were 65.32, 66.73 and 67.83 years. The distribution of patients according to age and log SCN showed no clear threshold values for age and sublimon accumulation. Dividing all patients into groups with ages < 65 and > 65 years also showed no significant difference in the log SCN (P = 0.53, R² = 0.01). No difference was observed in the AF group (P = 0.92, R² = 0.00).
Figure 3  Scatter plot of age and log SCN/cell in all subjects
The least-squares regression line is shown.

Figure 4  Scatter plot of LAD and log SCN/cell in all subjects
The least squares regression line is shown.

**mtDNA sublimon and AF duration**

Among the AF group, five patients were excluded from this analysis, as their AF onset was unknown. In the remaining patients (n = 33), there was no significant correlation (P = 0.661, R² = 0.01) between log SCN and duration of AF.

**mtDNA sublimon and LAD**

LAD was significantly higher in the AF group than in the SR group (AF group, 5.74 ± 0.86 cm, n = 38; SR group, 4.17 ± 0.44 cm, n = 28; P < 0.0001, R² = 0.55). The correlation between log SCN and LAD in all patients was not statistically significant (P = 0.560, R² = 0.1; Figure 4). Studying the relationship between LAD and log SCN in the AF and SR groups separately showed no significant correlation in either group (P = 0.171, R² = 0.05 in AF, and P = 0.943, R² = 0.00 in SR).

**mtDNA sublimon in POAF in the SR group**

Fifteen out of 35 (42.8%) SR patients developed AF in the post-operative period (within 72 h). The majority of these patients required medication to terminate AF. Patients who developed AF did not have a significantly different (P = 0.52, R² = 0.01) log SCN (2.68 ± 0.46) compared with those who remained in SR (2.81 ± 0.67). The mean age for the POAF group was 66.1 ± 8.6 years compared with 62.6 ± 9.5 years in the non-POAF patients, but this difference was not significant (P = 0.27, R² = 0.04).

**mtDNA sublimon and the type of surgery**

The 73 patients involved in this study were distributed with regard to the underlying disease and type of surgery as shown in Table 1. Studying these groups showed no effect of the underlying disease on SCN (P = 0.94, R² = 0.02).

**Effect of drug therapy on mtDNA deletion in the SR group**

Patients in the SR group were taking several medications continuously in the pre-operative period. These medications included statins, ACE (angiotensin-converting enzyme) inhibitors or AII (angiotensin II) antagonists, calcium-channel blockers, β-blockers, aspirin, nitrates and diuretics (mainly frusemide). In these patients there was no convincing evidence of an effect of medication on the SCN (Table 2). There was also no difference in log SCN between patients on medication who developed POAF and those who did not in this group (Table 3).
Table 3 Comparison of the log SCN values in patients in the SR group on medication and without or with POAF

<table>
<thead>
<tr>
<th>Medication</th>
<th>Log SCN ± S.D.</th>
<th>n</th>
<th>Log SCN ± S.D.</th>
<th>n</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients with POAF</td>
<td></td>
<td></td>
<td>Patients without POAF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Statins</td>
<td>2.59 ± 0.26</td>
<td>7</td>
<td>2.51 ± 0.24</td>
<td>10</td>
<td>0.51</td>
</tr>
<tr>
<td>ACE inh + AIIA</td>
<td>2.54 ± 0.24</td>
<td>6</td>
<td>2.71 ± 0.38</td>
<td>8</td>
<td>0.35</td>
</tr>
<tr>
<td>Calcium-channel blocker</td>
<td>2.95 ± 0.87</td>
<td>9</td>
<td>2.89 ± 0.57</td>
<td>7</td>
<td>0.85</td>
</tr>
<tr>
<td>β-Blocker</td>
<td>2.76 ± 0.59</td>
<td>7</td>
<td>2.83 ± 0.07</td>
<td>10</td>
<td>0.85</td>
</tr>
<tr>
<td>Aspirin</td>
<td>2.72 ± 0.47</td>
<td>11</td>
<td>2.68 ± 0.58</td>
<td>8</td>
<td>0.88</td>
</tr>
<tr>
<td>Nitrates</td>
<td>2.79 ± 0.54</td>
<td>8</td>
<td>2.91 ± 0.95</td>
<td>8</td>
<td>0.76</td>
</tr>
<tr>
<td>Diuretics</td>
<td>2.66 ± 0.37</td>
<td>10</td>
<td>2.46 ± 0.20</td>
<td>4</td>
<td>0.23</td>
</tr>
</tbody>
</table>

Gender and mtDNA sublimon

Among the 73 patients involved in the study, there were 42 (57.5%) males and 31 (42.5%) females. There was no difference (P = 0.431, R² = 0.01) in log SCN between males (2.97 ± 0.67) and females (2.84 ± 0.72).

DISCUSSION

To our knowledge, the present study is the first in which PCS abundance has been evaluated in AF patients. The importance of our present study comes from investigating the link between sublimons and AF in living atrial tissue; however, we were unable to identify a specific level of mtDNA sublimon and AF duration. The role for mtDNA mutation in some disorders, but this correlation was supported by the authors when they later studied sublimons in the heart [14]. The difference between our finding and those of Kajander et al. [13], but this correlation was supported by the authors when they later studied sublimons in the heart [14]. The difference between our finding and those of Kajander et al. [13,14] could be attributed to the different populations and tissues studied.

mtDNA sublimon and AF duration

In our present study, there was no significant association between log SCN and AF duration. The role of duration, independent of the underlying aetiology, in the progressive nature of AF is well established. This was
observed in paroxysmal AF, as transition to chronic fibrillation occurred in 31% of patients with paroxysms shorter than 2 days compared with 46% when the episodes of AF were longer in duration [30]. Moreover, the success rate of cardioversion in AF patients receiving serial cardioversion therapy was found to be higher when AF duration was shorter than 36 months [31]. However, this was not sufficiently supported on the molecular level in this study.

mtDNA sublimon and LAD

The lack of a correlation between log SCN and LAD leads us to conclude that mtDNA damage is not part of the atrial myopathy in AF. The insignificant role for mitochondrial sublimons in our present study is consistent with previous findings in cardiac tissue [14].

The association of atrial dilatation and AF is well documented by both invasive and non-invasive techniques. This may be explained by the structural changes in atrial tissue in AF, since previous studies have reported [7,8] that left atrial enlargement is associated with interstitial fibrosis. In valvular AF with marked left atrial enlargement, the muscle fibres are replaced by microscopic scar tissue [7]. A dilation of both atria with an increase in the ratio of fibrous tissue to muscle in most cases of AF of greater than 1 month duration was reported [8]. Cellular substructural changes in atrial arrhythmias were described, including loss of myofilaments, presence of glycogen granules, accumulation of sarcoplasmic reticulum-like material and aggregates of small mitochondria [32].

In dogs subjected to sustained atrial pacing, biatrial enlargement was observed with the ability to induce AF [33]. Pathologically there were changes, including disruption of the sarcoplasmic reticulum and giant mitochondria. In a goat model of AF [6], the mitochondria were slender and elongated. Alterations in the myofilaments, sarcoplasmic reticulum and mitochondria were also found as focal degenerative changes in patients with an atrial septal defect and atrial dilatation [34].

In the above studies, it was suggested that left atrial dilatation can be a precursor to, as well as a consequence of, AF. Although the mitochondrial changes were observed, none of these studies discussed the potential role for mitochondria and mtDNA in the pathogenesis of AF.

The present study examined changes at the molecular level in order to unmask a possible role of the dysfunctional mtDNA in AF, based on previous observations. The work of Aime-Sempe et al. [35] showed that in fibrillating and dilated atria the apoptotic process and myolysis contribute to cellular remodelling. The role of mtDNA in apoptosis is well established [36]; however, the findings in our present study did not support a role for mtDNA in the pathological changes accompanying AF.

Study limitations

It would have been more appropriate for the present study to obtain the left atrial appendage rather than RAA, since functional and structural changes are linked to AF.

The PCR methods used in the present study are specifically designed to investigate only one type of mtDNA rearrangement, i.e. 3.75 kb partial duplication. This does not provide a complete mutational profile of the entire mitochondrial genome, where other mtDNA mutations/deletions may also exist.

Conclusion

The present study suggests that the role of mtDNA deletion in pathophysiology of AF is probably neutral or at least is unlikely to be a major contributing factor in the occurrence of AF.

ACKNOWLEDGMENTS

We thank Ian Holt and Carrie Turner of the MRC Dunn Human Nutrition Unit, Cambridge, U.K. for kindly providing the ρ° cell line DNA for this study. We also acknowledge grant support from the Western Garfield Foundation.

REFERENCES


© 2004 The Biochemical Society


Received 23 July 2003/4 February 2004: accepted 12 February 2004

Published as Immediate Publication 12 February 2004, DOI 10.1042/CS20030252