Biochemical and Biophysical Research Communications 407 (2011) 23–27

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Mutational hotspots in the mitochondrial genome of lung cancer

So-Jung Choi [a], Sung-Hyun Kim [a], Ho Y. Kang [e], Jinseon Lee [a], Jong H. Bhak [e], Insuk Sohn [b], Sin-Ho Jung [b,g], Yong Soo Choi [c], Hong Kwan Kim [c], Jungho Han [d], Nam Huh [f], Gyusang Lee [f], Byung C. Kim [e], Jhingook Kim [a,c,*]

[a] Cancer Research Center, Samsung Biomedical Research Institute, Samsung Medical Center, Sungkyunkwan University, School of Medicine, 50 Ilwon-Dong, Kangnam-Ku, Seoul, Korea
[b] Samsung Cancer Research Institute, Samsung Medical Center, Sungkyunkwan University, School of Medicine, 50 Ilwon-Dong, Kangnam-Ku, Seoul, Korea
[c] Department of Thoracic Surgery, Samsung Medical Center, Sungkyunkwan University, School of Medicine, 50 Ilwon-Dong, Kangnam-Ku, Seoul, Korea
[d] Department of Pathology, Samsung Medical Center, Sungkyunkwan University, School of Medicine, 50 Ilwon-Dong, Kangnam-Ku, Seoul, Korea
[e] Theragen Bio Institute, Theragen Etex Co. Ltd., 443-270 Iui-Dong, Suwon, Gyeonggi, Korea
[f] Samsung Advanced Institute of Technology, Samsung Electronics Co. Ltd., Mt. 14-1 Nongseo-Dong, Giheung-gu, Yongin, Gyeonggi, Korea
[g] Department of Biostatistics and Bioinformatics, Duke University Medical Center, Durham, NC 27710, USA

ARTICLE INFO

Article history:
Received 10 February 2011
Available online 18 February 2011

Keywords:
Mitochondrial mutation
Lung cancer
Smoking
10398
8701
DCA

ABSTRACT

We determined the somatic mutations in the mitochondrial genomes of 70 lung cancer patients by pairwise comparative analyses of the normal- and tumor-genome sequences acquired using Affymetrix Mitochondrial Resequencing Array 2.0. The overall mutation rates in lung cancers were Approximately 100 fold higher than those in normal cells, with significant statistical correlation with smoking ($p = 0.00088$). Total of 532 somatic mutations were evenly distributed in 499 positions with very low overall frequency (1.07/bp), but the non-synonymous mutations causing amino acid substitution occurred more frequently (1.83/bp), particularly at two positions, 8701 and 10398 (10.5/bp) that code for ATPase6 and NADH dehydrogenase 3, respectively. Despite the randomness or even distribution of the mutations, these two mutations occurred together in 86% of the cases. The linkage between the two most frequent mutations suggests that they were selected together, possibly due to their cooperative role during cancer development. Indeed, the mutation at 10398 was shown by Canter, Pezzotti, and their colleagues in 2009, as a risk factor for breast cancer. In this study, we identified two potential biomarkers that might be functionally linked together during the development of cancer.

1. Introduction

Various human cancers accumulate mitochondrial mutations [3–5] whose role in cancer development has been suggested in a number of studies [3,6,7]. It is particularly so in lung cancer due to the exposure to an elevated level of reactive oxygen species (ROS), generated as combustion products of cigarette. Causal relationship is sequentially established from smoking, to ROS, mutations, and lung cancer has been well supported [8–11]. Furthermore, the mutagenic effect of smoking on the mitochondrial genome is much higher than on the nuclear genome [12,13], partly due to the physical proximity to the environmental ROS and less efficient DNA repair mechanisms in mitochondria [14]. In cancer cells, majority of the mutations are not eliminated because apoptosis is dysfunctional [4,5,15]; the mitochondrial mutation rate in lung cancer cells is dramatically higher [15,16] than those in normal cells [12,17,18].

Mitochondrial dysfunction has been postulated to render cancer cells resistant to apoptosis since the Warburg hypothesis [19–22]. To understand how cancer cells recognize mitochondrial dysfunction, we need to identify the underlying factors and interactions that switch off the mitochondrial respiration and switch on aerobic glycolysis, a phenomenon called the Warburg effect [19]. The high glucose metabolism in cancer cells is exploited in positron emission tomography (PET) by imaging the uptake of 2-^{18}F-2-deoxyglucose, a radioactive glucose analog [23,24]. Inhibition of the glycolytic pathway by dichloroacetic acid (DCA), 2-deoxy-D-glucose (2-DG) reactivated mitochondria and induced apoptosis in cancer cells [3,21,22]. Knockdown of M2-piruvate kinase, a nuclear factor for the Warburg effect, also induced similar effect [20]. A remaining question is how critical mitochondrial mutations are monitored and/or recognized to induce the Warburg effect.

In this study, we identified two mutational hotspots in the mitochondrial genomes in smokers' lung cancers. These two mutations are common germline polymorphisms belonging to the same haplogroup N [26]. Particularly, A10398G is a widely reported breast cancer risk factor in African American females [1,2] that induced resistance to apoptosis in cybrid assays [25]. Therefore,

* Corresponding author at: Department of Thoracic Surgery, Samsung Medical Center, Sungkyunkwan University, School of Medicine, 50 Ilwon-Dong, Kangnam-Ku, Seoul, South Korea. Fax: +82 11 822 2148 7385.
E-mail address: jkimsmc@skku.edu (J. Kim).

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doi:10.1016/j.bbrc.2011.02.078

these mutations might not simply associate with smoking and lung cancer, but might provide a missing molecular link between smoking and lung cancer.

2. Materials and methods

2.1. Patients and specimen collection

Non small cell lung cancer (NSCLC) specimens, adjacent normal lung tissues, and peripheral lymphocytes were collected from 70 patients (30 smokers, 31 non-smokers, and 9 undeclared) who underwent surgical resections at Samsung Medical Center between 1996 and 2008 (Supplement Table 1). The specimens were collected with prior consents from the patients abiding by the guidelines provided by the institutional review board. Tumor specimens were examined by pathologists to remove the necrotic region and the intervening stroma abiding by the World Health Organization histopathological criteria. After the pathological examination, the tumor masses were snap frozen and maintained in liquid nitrogen until mitochondrial DNA extraction. Specimen with tumor cell contents greater than 90% were selected for DNA preps.

2.2. Isolation of mitochondrial genomic DNA

The frozen tissue samples were micro-dissected and lightly stained with hematoxylin to identify the portion consisting of 90% or more cancer cells. The genomic DNA was extracted with DNeasy kit (Qiagen). The DNA quality was assessed with the use of a Nanodrop spectrometer (Nanodrop Technologies). Control DNA from matched peripheral lymphocytes was processed in the same manner.

2.3. Resequening the whole mitochondrial genome

Affymetrix GeneChip® Human Mitochondrial Resequencing Array 2.0 (Mitochip v2.0) was used as the primary method, and for the Hyper Variable Region I, II that contained the monomorphic stretches particularly in the regions 67–380 and 16060–16380, the sequences were determined by Capillary Sequencing using ABI3730. For the Mitochip experiments, the mitochondrial DNA was amplified by PCR, fragmented, labeled, and hybridized to the Mitochip following the manufacturer's protocol; 25 ng of genomic DNA was used as template to amplify the whole mitochondrial genome into three parts, using three sets of primers (Mito1F: ACATAGCACATTACAGTCAAATCCCTTCTCGTCCC, Mito1R: TGAGATTGTT TGGGCTACTGCTCGCAGTGC, Mito2F: TACTCAATCCTCTGATCAGGGT GAGCATC AAACTC, Mito2R: GCTTGGATTAAGGCGACAGCGATT TCT AGGATAGT, Mito3F: TCATTTTTATTGCCACAACTAACCTCCTCGGACTC, Mito3R: CGTGATGTCTTATTTA AGGGGAACGTGTGGGCTAT). After 30 cycles of PCR, the products were purified with a DNA Amplification Clean-up kit (Clontech, CA, USA), and quantified. After pooling the equimolar PCR Products (117.7 pmol), they were fragmented by DNase I treatment, and end labeled with Biotin by Terminal Deoxynucleotidyl Transferase. The Labeled DNA was hybridized with the Mitochip for 16 hours in excess of BSA, Oligonucleotide control, Herring Sperm DNA in the hybridization mixture. The Mitochip was washed in Fluidics Station, and stained with Streptavidin Phycoerythrin (SAPE), and scanned by Genechip Array scanner 3000 7G (Affymetrix) Scanner. Insertions and deletions in the Hyper Variable Region I, II were determined by 478 redundant fragments designed within the Array 2.0 by aligning them with the public database (http://code.open-bio.org). In addition, the Hyper Variable Region I, II, particularly the regions 67–380 and 16060–16380 were amplified by PCR primers HV1F (5′-caccattagcacccaaagct-3′), HV1R (5′-gaggatggtggtcaagggac-3′), HV2F (5′-ctcacgggagctctccatgc-3′), HV2R (5′-ctgttaaaagtgcataccgcca-3′), and the PCR products were sequenced by ABI 3730xl DNA Analyzer (Applied Biosystems, USA).

2.4. Mitochondrial mutation analyses

To analyze the Mitochip 2.0 data, ABACUS algorithm based software was used as provided by the manufacturer (Affymetrix Sequence Analysis Software 4.1). The sequence data were analyzed by the Sequencing analysis 5.2 (Applied Biosystems, USA), Sequence Scanner (Applied Biosystems, USA), and ChromasPro (Technelysium Pty. Ltd.), BioEdit (version 7.0.9). To define the germline polymorphisms, revised Cambridge Reference Sequence (rCRS, GenBank accession NC_012920) was used as the reference sequences and to determine the cancer specific somatic mutations, the sequences from the normal lymphocytes were used as the reference sequences. In the cases of heteroplasmy with low prevalence, the sequence call could not be made by the manufacturer's protocol, and therefore, excluded from further mutation analyses. The difference of individual mutation counts between pre-defined sample groups according to the clinical quantitative factors was examined by one-way ANOVA (Analysis Of Variance). The differences were deemed significant at the 95% confidence interval. Chi-square tests were carried out to examine the association between mtDNA mutation and clinic-pathological features. In order to obtain the p-values for the chi-square tests that account for a possible dependency among the mutations within each gene, 100,000 permutations were carried out for each p-value. To test if the cancer mutations had some hidden rules that made statistically identifiable sample groups, a genetic clustering algorithm, STRUCTURE [27], was carried out. The method allows both the estimation of the number of genetically distinct clusters (K) and the assignment of individual patients to these clusters based on their genotypes. The algorithm of STRUCTURE assigns the data to a randomly-mating ancestral population K. After the K specification, independent runs for several choices of K are computed. As input genotype data, two individual variation matrices were made. One used a gene-based mutation model—i.e., if a gene had a mutation compared to rCRS and its mutation base-frequency was lower than 3% in the 70 Korean blood samples, it was encoded as "1" otherwise "0". The other model encoded mutations as "1" different from the rCRS sequence. Simulation was made from K = 2 to K = 7 with an admixture model, an allele frequency correlation model, a 1000 burn-in and 1000 Markov chain Monte Carlo repetitions.

3. Results

3.1. Distribution of somatic mutations in the mitochondrial genome

The somatic mutations in the mitochondrial genomes of 70 Korean NSCLC patients were charted, by comparative analyses of the sequences from the tumor and the matched normal lymphocytes. The sequences of the Hyper Variable Regions and all the non-synonymous mutations were confirmed by direct sequencing. 532 somatic mutations were found in a total of 499 positions, or 3.03% of the whole mitochondrial genome. To show the distribution of mitochondrial mutations throughout the mitochondrial genome, the frequencies of all mitochondrial mutations per unit length were calculated (Table 1), color-coded in gray scale, and all the 97 non-synonymous mitochondrial mutations were marked as dots on the genome (Fig. 1). The highest frequency was observed in the D-loop region, particularly in the hyper-variable sequences 1 (2.83 mutations/kb/person) followed by hyper-variable sequences 2 (1.31 mutations/kb/person), and the lowest frequency was found in tRNAs (0.2 mutations/kb/person). The average muta-

Table 1
Frequency of the mitochondrial mutations.

Region	Total MM[1]s (MMs/kb/person)	Non-Syn[2] (MMs/kb/person)	% Non-Syn (Non-Syn/Total MMs) 100
D-loop HVS1	2.83	0	N/A[3]
D-loop HVS2	1.31	0	N/A
ATP6	**0.48**	**0.38**	**78.26**
ND3	**1.28**	**0.54**	**42.06**
ND2	0.63	0.26	41.34
ATP8	0.55	0.21	37.5
ND1	0.22	0.07	33.41
CO1	0.29	0.09	32.26
ND5	0.32	0.08	24.39
CO3	0.38	0.07	19.07
ND4	0.29	0.05	17.87
CYB	0.63	0.08	12.01
CO2	0.21	0.02	10
ND6	0.38	0.03	7.14
ND4L	0.19	0.1	1.31
12S	0.3	0	N/A
16S	0.15	0	N/A
Thr	0.87	0	N/A
Cys	0.65	0	N/A
Arg	0.44	0	N/A
Asp	0.21	0	N/A
Met	0.21	0	N/A
Glu	0.21	0	N/A
Gln	0.2	0	N/A

MM: mitochondrial mutation;
Non-Syn: non-synonymous mutation;
N/A: not applicable.

tional frequency in the lung cancer samples was 0.8 bp/kb which is approximately 100 fold higher than those in normal cells [12]. A total of 97 non-synonymous mutations were observed in 56 positions in 19 patients. The highest mutation frequencies were seen at 10398 (n = 11/19) and 8701 (n = 10/19) positions which accounted for the high non-synonymous mutation ratios in NADH dehydrogenase 3 and ATPase 6 (Table 1). Among the 19 patients with non-synonymous mitochondrial mutations, 17 (89.5%) had at least one mitochondrial mutation in respiratory chain complex 1 (RCC1). Taken together, these data suggested that mitochondrial mutations were most frequently mutated in the non-coding D-loop region, and the non-synonymous mutations causing amino acid substitution were concentrated in specific positions in RCC1 at 8701 and 10398.

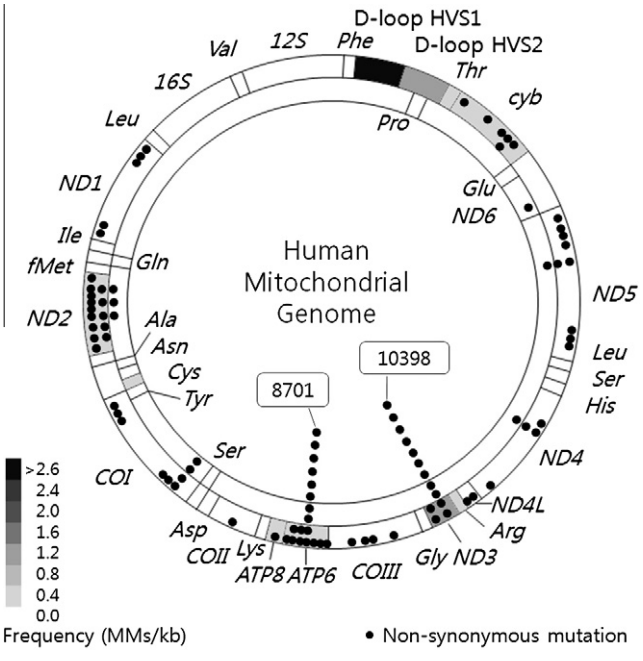

Fig. 1. Distribution of somatic mutations in the mitochondrial genome. Total 535 mutations were distributed in 499 positions (97 non-synonymous mutations in 56 positions). The mutational frequency was coded in gray scale (mutational frequency = the number of mutations/kb/person). Highest mutational frequencies were found in the regulatory D-loop regions, and non-synonymous mutations, as marked by black dots, were most frequently found at 8701 and 10398 positions.

3.2. Association between the somatic mutations and smoking

Among 532 somatic mitochondrial mutations, 452 (88%) occurred among smokers (n = 30, average 34.8 years of smoking), and 63 (12%) occurred among non-smokers (n = 31). Among the smokers, the number of mitochondrial mutations ranged from 50 to none (average = 6.5 mitochondrial mutations/person, variance = 326). Among the non-smokers, the number of mitochondrial mutations ranged from 28 to none (average = 0.9 mitochondrial mutations/person, variance = 34). Association between smoking and the amount of mitochondrial mutations was tested by ANOVA (p = 0.00088, Table 2), and the association between single mitochondrial mutations and smoking was tested by multivariate logistic regression, which identified mitochondrial mutations at 10398 and 8701 significantly associated with smoking (p = 0.02254 and 0.00583, respectively). In order to search for mitochondrial mutations associated with other clinical parameters, such as age, gender, cell type, pStages, recurrence status, overall survival, the same statistical analyses were carried out, but no association was observed with any mitochondrial mutations. For example, the amount of mitochondrial mutations showed little association with the recurrence status (p = 0.589). To look for hidden clusters of patients that can be stratified by unknown rules of genes with mutations, a simulation test was carried out by the population structure analysis [27], as shown in Supplement Fig. 1. The tested ancestral population number was from 2–7 but it was not possible to identify any hidden population cluster based on the genes with somatic mutations. Therefore, smoking was the only clinical variable that showed significant association with the somatic mitochondrial mutations found in the samples tested.

4. Discussion

Smoking is a well known cause of lung cancer [8] and mitochondrial mutations [9,10], but the causal relationship between them and the underlying molecular mechanisms remain unexplored. In this study, we charted 532 somatic mitochondrial mutations in 70 Korean NSCLCs. Compared with the normal mutation rate in human mitochondrial genome [12], these somatic mutations are comparable with the polymorphisms that have accumulated over the past 20,000–25,000 years of human evolution since the divergence between Korean (Mongolian) and European populations [28]. Question is whether any of them was selected for the survival of the cancer cells, and our data suggest that two specific mutations at 8701 and 10398 might be, as the mutation frequency was significantly higher in these positions. It is still possible that they are simply permissive non-lethal mutations, and they were accumulated as they were not selected against. An example of such permissive mutations might be the synonymous mutations that do not cause amino acid substitution. The frequency of a non-synonymous mutation relative to the synonymous mutations within the gene might help neutralizing the permissive variable on the mutation frequency, and therefore, might be a better way to estimate the selective force imposed on

Table 2
ANOVA association of somatic mitochondrial mutations with smoking.

Groups	Counts		Sum	Average		Variance
Smoking	30		418	13.93333		326.0644
No smoking	31		61	1.967742		34.03226
Source of variation	SS	df	MS	F	*p*-Value	F crit
Between groups	2182.838	1	2182.838	12.29259	0.000876	4.003982
Within groups	10476.83	59	177.5735			
Total	12659.67	60				

SS: sum of squares;
df: degrees of freedom;
MS: mean of sum of square;
F: F-ratio;
F crit: F critical one-tail.

the mutation. The ratios of non-synonymous mitochondrial mutations over the synonymous mutations in ATPase6 and ND3 were 78% and 42% (Table 2), which is significantly higher than the average ration of 18% throughout the whole mitochondrial genome. Mutations at 10398 and 8701 positions were the most frequent non-synonymous mitochondrial mutations found at in ND3 and ATPase6 coding regions. Among the 19 patients with non-synonymous mitochondrial mutations, 10 and 11 had mutations at 8701 and 10398, respectively, and 17/19 had at least one mutation in RCC1 including the two mutations at 8701 and 10398. We also found the highest mitochondrial mutation frequencies in the regulatory HVS1 in D-loop region and ND3 coding region, consistent with previous reports [4,5]. In this study we charted somatic mutations in lung cancer, and identified two non-synonymous mutations with significantly higher frequencies.

Functional studies have shown that NADH dehydrogenase generated mitochondrial oxidative stress [29], or might be involved in controlling apoptosis [30]. Interestingly, the two mitochondrial mutations at 10398 and 8701 are commonly found germline polymorphisms that belong to the same haplogroup N [26], and such a close linkage was also observed in our somatic mutation data; 9/10 patients with mutation at 8701 had mutation at 10398, and 9/11 patients with mutation at 10398 had mutation at 8701. Considering the randomness in somatic mutations, this linkage between the two somatic mutations suggests a functional relationship between them. Furthermore, the germline polymorphisms at 10398 have been widely reported as a risk factor for breast cancer [1,2], and a functional study showed that it rendered cells resistant to apoptosis [25]. 10398G/8701G and 10398A/8701A cybrids cell lines showed differential cytosolic calcium signaling effects [30]. Therefore, at least one of the two somatic mutations has been studied extensively for the role in carcinogenesis.

Association between mitochondrial mutations and clinical variables was tested by ANOVA and multivariate logistic regression, which identified that smoking is significantly associated with the amount of somatic mitochondrial mutations ($p = 0.00088$, Table 2) and the two specific mutations at 10398 ($p = 0.02254$) and 8701 ($p = 0.00583$). To look for hidden clusters of patients that can be stratified by unknown rules of mutated genes, population structure analysis was carried out, but did not identify any rule, supporting that smoking was the only clinical variable that showed significant association with the somatic mitochondrial mutations in the samples tested. The relatively high variances in Table 2 are due to the wide range of mutations from 0 to 50 per person. Specifically, both smokers and non-smokers can be largely divided into two subgroups: one with either almost no mutation (0–3), and another with dozens of mutations per person (Supplement Fig. 2). However, the smoke-related mutations did not show association with overall survival, suggesting that smoke-related lung cancers are no more malignant than the others. Nonetheless, the role of these mitochondrial mutations needs to be explored, as it might shed a light on how cancer avoid apoptosis, and who might benefit from the mitochondria targeted therapy, such as DCA.

In this study, we charted mitochondrial somatic mutations in 70 lung cancer patients, and found that significantly more (7 fold) mutations accumulated in smokers than in non-smokers, and identified two mutational hotspots at 8701 and 10398 as potential biomarkers for lung cancer. One of them at 10398 is already known as a germline risk factor for breast cancer. Further molecular studies on these mutations might shed a light on their role during cancer development.

Acknowledgments

This work was supported by Samsung Biomedical Research Institute grant, #SBRI C-A6-411, C-A7-802, and the National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) (No. R01-2006-000-11114-0, and NTX2091113), and Samsung Advanced Institute of Technology, Samsung Electronics Co. Ltd. The funding sources were not involved in the study design, interpretation of the data, or in submission for publication.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.02.078.

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