ROS-Generating Mitochondrial DNA Mutations Can Regulate Tumor Cell Metastasis

Ishikawa Kaori, Takenaga Keizo, Akimoto Miho, Koshikawa Nobuko, Yamaguchi Aya, Imanishi Hirotake, Nakada Kazuto, Honma Yoshio, Hayashi Jun-Ichi

Journal or Publication Title: Science
Volume: 320
Number: 5876
Page Range: 661-664
Year: 2008-05

Copyright 2008 by the American Association for the Advancement of Science

URL: http://hdl.handle.net/2241/101575
doi: 10.1126/science.1156906
ROS-generating Mitochondrial DNA Mutations Can Regulate Tumor Cell Metastasis

Kaori Ishikawa1, 2, 3*, Keizo Takenaga4, 5*, Miho Akimoto5, Nobuko Koshikawa4, Aya Yamaguchi1, Hirotake Imanishi1, Kazuto Nakada1, 2, Yoshio Honma5, and Jun-Ichi Hayashi1†

1Graduate School of Life and Environmental Sciences, 2TARA Center, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan.

3Japan Society for the Promotion of Science (JSPS), 8 Ichiban-cho, Chiyoda-ku, Tokyo 102-8472, Japan.

4Division of Chemotherapy, Chiba Cancer Center Research Institute, 666-2 Nitona, Chuo-ku, Chiba 260-8717, Japan.

5Shimane University Faculty of Medicine, 89-1 Enya-cho, Izumo, Shimane 693-8501, Japan.

* These authors contributed equally to this work.

† To whom correspondence should be addressed. E-mail: jih45@sakura.cc.tsukuba.ac.jp
Abstract

Mutations in mitochondrial DNA (mtDNA) occur at high frequency in human tumors, but whether these mutations alter tumor cell behavior has been unclear. We used cytoplasmic hybrid (cybrid) technology to replace the endogenous mtDNA in a mouse tumor cell line that was poorly metastatic with mtDNA from a cell line that was highly metastatic – and vice versa. Using assays of metastasis in mice, we found that the recipient tumor cells acquired the metastatic potential of the transferred mtDNA. The mtDNA conferring high metastatic potential contained G13997A and 13885insC mutations in the gene encoding NADH dehydrogenase subunit 6 (ND6). These mutations produced a deficiency in respiratory complex I activity and were associated with overproduction of reactive oxygen species (ROS). Pretreatment of the highly metastatic tumor cells with ROS scavengers suppressed their metastatic potential in mice. These results indicate that mtDNA mutations can contribute to tumor progression by enhancing the metastatic potential of tumor cells.
Because most chemical carcinogens bind preferentially to mtDNA rather than to nuclear DNA (1-3), mtDNA is considered to be their major cellular target. It has been hypothesized that the resultant somatic mutations in mtDNA play a causal role in oncogenic transformation (3). Many subsequent studies have supported the idea of preferential accumulation of somatic mutations in tumor mtDNAs (4-9) and their contribution to tumor growth (10, 11). However, the apparent high frequency of mtDNA mutations in tumors could simply be due either to their stochastic accumulation (12, 13) or to laboratory errors (14). Moreover, if mtDNA mutations induce oncogenic transformation, all the offspring of a mother carrying such mutations should develop tumors due to the maternal inheritance of mtDNA (15, 16), but no bias toward maternal inheritance of tumor development has been reported. Nonetheless, it remains possible that mtDNA mutations are involved at a later stage of tumorigenesis – for example, in the development of metastatic potential. Recent studies demonstrated that dysfunction of the tricarboxylic acid cycle (TCA cycle) caused by mutations in nuclear DNA controls tumor phenotypes by the induction of a pseudo-hypoxic pathway under normoxic conditions (17-19). However, there has been no evidence of the involvement of mtDNA mutations in the development of metastatic potential or in the regulation of the pseudo-hypoxic pathway, because of the difficulty of excluding possible involvement of nuclear DNA mutations in these processes (20).

Here we have examined the role of pathogenic mtDNA mutations in the development of tumor cell metastasis by studying two mouse tumor cell lines with different metastatic potentials (low metastatic P29 and high metastatic A11 cells), that originated from Lewis lung carcinoma (Table S1; 21-23). We compared mitochondrial respiratory function by
estimating the activities of respiratory complexes, and found that P29 cells had normal activities, whereas A11 cells showed reduced activity of complex I (NADH dehydrogenase) (Fig. 1A). Complex I defects were also observed in high metastatic fibrosarcoma B82M cells, but not in high metastatic colon adenocarcinoma LuM1 cells (Fig. 1A), suggesting that all metastatic tumors are not always associated with complex I defects.

Since complex I consists of subunits encoded by both nuclear DNA and mtDNA (24), it was necessary to determine which genome, nuclear or mitochondrial, was responsible for the complex I defects, and whether the complex I defects were responsible for the high metastatic potential. We addressed these issues by complete reciprocal exchange of mtDNAs between P29 and A11 cells by means of cell fusion to isolate trans-mitochondrial cybrids (Table S2, Fig. S1A), and examined whether complex I defects and metastatic potentials were co-transferred with the mtDNA. The results showed that complex I activity decreased in the cybrids with A11 mtDNA, whereas those with P29 mtDNA showed normal activity, irrespective of whether their nuclear DNAs were derived from P29 or A11 cells (Fig. 1B). Thus, complex I defects in the cybrids with A11 mtDNA appear to result from pathogenic mutations in their mtDNA, not in their nuclear DNA. We then examined the metastatic potential of the cybrids by inoculating them into a tail vein (to test “experimental” metastasis) and under the skin (to test “spontaneous” metastasis) of C57BL/6 mice, and counting the number of nodules formed in the lung. Cybrids with A11 mtDNA acquired high metastatic potential, whereas cybrids with P29 mtDNA lost metastatic potential (Table S2). These observations suggest that complex I defects and high metastatic potential are transferred simultaneously with the transfer of mtDNA from the
A11 cells, whereas normal complex I activity and low metastatic potential are transferred simultaneously with the transfer of mtDNA from P29 cells. The mtDNA of A11 cells is therefore likely to harbour a mutation(s) responsible for complex I defects and metastasis.

We next examined whether these findings could be generalized to additional tumor cell lines. In these experiments, we transferred mtDNA from A11 cells into fibrosarcoma B82 cells with low metastatic potential and normal complex I activity, resulting in isolation of B82mtA11 cybrids (Table S2). Conversely we transferred mtDNA from B82M cells, which are derived from B82 cells but express high metastatic potential and complex I defects, into low metastatic P29 cells, resulting in isolation of P29mtB82M cybrids. Both B82mtA11 and P29mtB82M cybrids acquired complex I defects (Fig. 1C) and high metastatic potential (Table S2), suggesting the co-transfer of these phenotypes and the mtDNAs from high to low metastatic cells of different tumor types. Notably, transfer of mtDNA from high metastatic A11 and B82M cells into non-transformed NIH3T3 cells did not induce tumorigenicity and metastatic potential in the resultant NIHmtA11 and NIHmtB82M cybrids (Table S2, Fig. S2A). Thus, pathogenic mtDNA mutations that induce complex I defects are present in A11 and B82M cells and control development of metastases; however, these mutations do not control the development of tumorigenicity and metastasis, at least in non-transformed NIH3T3 cells.

To identify the pathogenic mtDNA mutations that induced complex I defects and high metastatic potential in A11 and B82M cells, we compared the whole mtDNA sequences between P29 and A11 cells, and between B82 and B82M cells. We conclude that
a missense G13997A mutation in the A11 cells and a frame-shift 13885insC mutation in the B82M cells, both within the ND6 (NADH dehydrogenase subunit 6) gene, are the pathogenic mutations that induce complex I defects, since these are the only mutations exclusively observed in the mtDNA of the high metastatic A11 cells and B82M cells (Table 1). Restriction enzyme digestion of the PCR products amplified using mismatched primers suggests complete and reciprocal replacement of parental mtDNAs in our cybrids (Fig. S3).

We next explored how the mutated mtDNA and resultant complex I defects regulate metastasis. Since complex I defects may lead to overproduction of ROS (24, 25), we estimated the amounts of ROS (Fig. S4), and found that the cybrids with the mutated mtDNA from A11 cells showed enhanced ROS production, whereas the cybrids without the mutated mtDNA from P29 cells did not (Fig. S4B). Such co-transfer of ROS-producing properties to the cybrids along with the transfer of mtDNA with or without the mutation suggests that ROS overproduction is due to the G13997A mutation. ROS overproduction was also observed in the P29mtB82M and B82mtA11 cybrids (Fig. S4C).

How does ROS overproduction regulate metastasis, and which nuclear genes (if any) are involved in this process? We have reported previously (22, 26) that A11 cells, but not P29 cells, show resistance to hypoxia-induced apoptosis, accompanied by upregulation of antiapoptotic MCL-1 (myeloid cell leukemia-1). Moreover, A11 cells showed higher expression levels of two genes associated with neoangiogenesis, HIF-1α (hypoxia-inducible factor-1α) and VEGF (vascular endothelial growth factor), in comparison of P29 cells (27). Thus, we focused here on the expression of these three nuclear-coded genes. We
found that upregulation of the MCL-1, HIF-1α, and VEGF was co-transferred when mutant mtDNA was transferred from A11 cells to the P29mtA11 and A11mtA11 cybrids. Down regulation of three genes was co-transferred when wild-type mtDNA was transferred from P29 cells to the P29mtP29 and A11mtP29 cybrids (Fig. 2). Therefore, the mutated mtDNA and the resultant complex I defects induce upregulation of the MCL-1, HIF-1α, and VEGF genes and are associated with high metastatic potential (Fig. S1B). Gene expression profiling to compare P29mtP29 with P29mtA11, and A11mtP29 with A11mtA11 showed consistent upregulation of other genes possibly related to metastasis in the cybrids with A11 mtDNA (Table S3), suggesting involvement of additional genes in the mtDNA-mediated effects on metastasis.

To obtain direct evidence that ROS overproduction caused by the mutated mtDNA from A11 cells is responsible for high metastatic potential, we treated the P29mtA11 cybrids with ROS scavengers, and examined their effects on the amounts of ROS and on the expression of the genes and the phenotypes related to metastasis. N-acetylcysteine (NAC), which has been used as an anti-cancer agent in preclinical models, was used as one ROS scavenger. The results showed that treatment of the cybrids with NAC in cell culture reduced the amount of ROS (Fig. 3A) and downregulated MCL-1 (Fig. 3B). Moreover, pre-treatment of the cybrids with NAC reduced their metastatic potential in two mouse models (Fig. 3C). Similar results were obtained by treatment with another ROS scavenger, Ebselen, which is a mimic of glutathione peroxidase (Fig. 3). Thus, ROS overproduction caused by the mutated mtDNA induces a high metastatic potential, at least in part, by upregulation of MCL-1. This idea is supported by the finding that down-regulation of MCL-1 in
P29mtA11 cybrids by siRNA also suppressed their metastatic potential (Fig. S5). Moreover, NAC treatment suppressed the metastatic potential without reducing glycolytic activity (Fig. S6), suggesting that metastasis is not caused by upregulation of glycolysis.

Contribution of mtDNA to tumor cell metastasis can be extended to human tumors, since the transfer of mtDNA from human breast cancer MDA-MB-231 cells expressing high metastatic potential into low metastatic HeLa cells induces complex I defects, increased ROS production, and high metastatic potential in HeLa cells (Fig. 4). These observations suggest that the mtDNA in MDA-MB-231 cells can promote metastasis, although we have not done the mtDNA sequencing. Therefore, the metastatic potential of all mouse and human tumor cell lines we examined was greatly enhanced by exchanging their endogenous mtDNA with mutant mtDNA that induces complex I-mediated ROS overproduction. Recent reports showed that a pathogenic mutation in the ATP6 gene of human mtDNA generated ROS and enhanced tumor growth (10, 11). However, in our experiments, the enhanced growth rate of primary tumors did not necessarily correlate with expression of the high metastatic potential in mouse tumors (Fig. S2B).

This study partially resolves the debate on the relevance of mtDNA mutations in tumors (4-14) by showing that mutations in mtDNA can control the metastatic potential of certain tumor cells, but do not confer tumorigenic potential to non-transformed mouse NIH3T3 cells. Moreover, reversible regulation of metastasis by the exchange of mtDNA between P29 and A11 cells and by treatment with ROS scavengers suggests that metastasis of these cells is regulated by ROS-mediated reversible upregulation of nuclear genes, but not by ROS-mediated acceleration of genetic instability. The mtDNA-mediated reversible
control of metastasis, therefore, reveals a novel function of mtDNA, and suggests that in such cases ROS scavengers may be therapeutically effective in suppressing metastasis.

References and Notes


23. Materials and methods are available as supporting material on *Science* Online.


28. All animal experiments were performed in compliance with the institutional guidelines (Chiba Cancer Center and University of Tsukuba) for the care and use of laboratory animals.
29. This work was supported by Grants-in-Aid for Scientific Research (S) from the Japan Society for Promotion of Science (JSPS), by Grants-in-Aid for Creative Scientific Research from JSPS, and by Grants-in-Aid for Scientific Research on Priority Areas from The Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT) to J.-I.H. This work was also supported by grants for a Research Fellowship from JSPS for Young Scientists to K.I. and by Grants-in-Aid for Third Term Comprehensive Control Research for Cancer from the Ministry of Health, Labour, and Welfare and for Scientific Research from MEXT to K.T.

Supporting Online Material

www.sciencemag.org/cgi/content/full/

Materials and Methods

Figs. S1 to S6

Tables S1 to S3

References
Table 1  Identification of pathogenic mutations by comparison of mtDNA sequences between low and high metastatic mouse tumor cells

<table>
<thead>
<tr>
<th>Position</th>
<th>Gene</th>
<th>Amino acid change</th>
<th>Mouse strain</th>
<th>C57BL/6</th>
<th>P29</th>
<th>A11</th>
<th>L929</th>
<th>B82</th>
<th>B82M</th>
<th>NIH/3T3</th>
</tr>
</thead>
<tbody>
<tr>
<td>T6589C</td>
<td>COI</td>
<td>V421A</td>
<td>T</td>
<td>T</td>
<td>T</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>T</td>
</tr>
<tr>
<td>G9348A</td>
<td>COIII</td>
<td>V248I</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>G</td>
</tr>
<tr>
<td>T9461C</td>
<td>ND3</td>
<td>Silent</td>
<td>T</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>9821-PolyA</td>
<td>tRNA^Arg</td>
<td>8A</td>
<td>9A</td>
<td>9A</td>
<td>10A</td>
<td>10A</td>
<td>10A</td>
<td>10A</td>
<td>10A</td>
<td>10A</td>
</tr>
<tr>
<td>C11493A</td>
<td>ND4</td>
<td>P443T</td>
<td>C</td>
<td>A</td>
<td>A</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>A13672T</td>
<td>ND6</td>
<td>Silent</td>
<td>A</td>
<td>T</td>
<td>T</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>13885insC</td>
<td>ND6</td>
<td>Frame-shift</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>C*</td>
<td>-</td>
</tr>
<tr>
<td>G13997A</td>
<td>ND6</td>
<td>P25L</td>
<td>G</td>
<td>G</td>
<td>A*</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
</tr>
</tbody>
</table>

Accession No. | AY172335 | EU312160 | EU312161 | AJ489607 | EU315229 | EU315228 | AY999076

* The G13997A mutation in ND6 is a missense mutation that changes the amino acid proline to leucine at a site that is highly conserved throughout vertebrates. The 13885insC mutation in ND6 is a frame-shift mutation that has been previously reported as a pathogenic mutation that induces significant complex I defects in some sublines of an L929 fibroblast cell line and A9 cells (see Materials and Methods).
Figure Legends

Figure 1 Mitochondrial respiratory function of parental mouse cells and their trans-mitochondrial cybrids.

(A) Comparison of respiratory complex activities between low and high metastatic tumor cell lines. P29 and A11 cells are low and high metastatic Lewis lung carcinoma cells, respectively; B82 and B82M cells are low and high metastatic fibrosarcoma cells, respectively; NM11 and LuM1 cells are low and high metastatic colon cancer cells, respectively (see Table S1). Respiratory complex I (NADH dehydrogenase), complex II (succinate dehydrogenase), and complex III (cytochrome c reductase) are components of the electron-transport chain located in the mitochondrial inner membrane. Mitochondrial respiratory function was examined by estimating their activities. Since the complexes II+III activity is normal in the A11 and B82M cells, the reduced activity of complexes I+III exclusively observed in the A11 and B82M cells should represent complex I defects. (B) Comparison of respiratory complex activities of the cybrids with mtDNA exchanged between low metastatic P29 and high metastatic A11 Lewis lung carcinoma cells. (C) Comparison of respiratory complex activities of the cybrids with mtDNA transferred from different types of tumor cells expressing high metastatic potential. Bars represent the mean ± s.d. (n = 3). *, $P < 0.05$, **, $P < 0.01$. 
Figure 2 Reversible control of metastasis-related nuclear gene expression by mtDNA.

(A) Expressions of nuclear-coded MCL-1 and HIF-1α, and (B) VEGF under normoxia (N) and hypoxia (H). As loading controls in the Western blots, we used β-actin for MCL-1 and E2F-1 for HIF-1α (A). In (B), blue bars represent cybrids carrying mtDNA from P29 cells (P29mtP29 and A11mtP29), and red bars represent cybrids carrying mtDNA from A11 cells (A11mtA11 and P29mtA11). Bars represent the mean ± s.d. (n = 3). *, P < 0.01, **, P < 0.001.

Figure 3 Suppression of metastasis by treatment of the P29mtA11 cybrids with ROS scavengers.

(A) Effects of NAC and Ebselen treatments on the amounts of ROS. The P29mtA11 cybrids (1 × 10^6 cells) treated with 5 μM DCFH-DA were subjected to FACScan for quantitative estimation of ROS (H_2O_2). FACScan was carried out before (green) and after (yellow) 24 h treatment of the cybrids with 20 mM NAC or 20 μM Ebselen. (B) Effects of NAC and Ebselen treatments on MCL-1 expression. Western blot analysis of MCL-1 was carried out before and after the treatment of P29mtA11 cybrids with 20 mM NAC or 20 μM Ebselen for 4 days. β-Actin served as the loading control. (C) Effects of NAC and Ebselen treatments on metastatic potential. The P29mtA11 cybrids pretreated for 4 days with 20 mM NAC or with 20
μM Ebselen were injected into the tail vein of C57BL/6 mice to test the experimental metastatic potential. To examine the effect of NAC administration on spontaneous metastatic potential, P29mtA11 cybrids without NAC pretreatment were injected subcutaneously into C57BL/6 mice, which subsequently received 10 mg/ml NAC in drinking water *ad libitum*. Bars represent the mean ± s.d. (n = 3). *, $P < 0.05$, **, $P < 0.01$.

**Figure 4** Induction of high metastatic potential in HeLa cells by introduction of mtDNA from human breast adenocarcinoma MDA-MB-231 cells expressing high metastatic potential.

Induction of (A) complex I defects, (B) ROS overproduction, and (C) high metastatic potential in the Hemt231 cybrids. HemtHe, HemtHe cybrids carrying nuclear DNA from $\rho^0$ HeLa cells and mtDNA from wild-type HeLa cells; Hemt231, Hemt231 cybrids carrying nuclear DNA from $\rho^0$ HeLa cells and mtDNA from MDA-MB-231 cells. Bars represent the mean ± s.d. (n = 3). *, $P < 0.05$. 
Figure 1

A

Lewis lung carcinoma

Fibrosarcoma

Colon cancer

P29 A11

B82 B82M

NM11 LuM1

B

Cybrid clones

P29mtP29 P29mtA11

A11mtP29 A11mtA11

C

Cybrid clones

P29mtP29B82M

B82mtB82

B82mtA11

NIHmtNIH

NIHmtB82M
Figure 2

A

<table>
<thead>
<tr>
<th>Nuclear DNA</th>
<th>mtDNA</th>
<th>P29</th>
<th>P29</th>
<th>A11</th>
<th>A11</th>
</tr>
</thead>
<tbody>
<tr>
<td>P29</td>
<td>P29</td>
<td>A11</td>
<td>A11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCL-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCL-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIF-1α</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2F-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIF-1α</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2F-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

VEGF (pg/10⁵ cells/24 h)

<table>
<thead>
<tr>
<th></th>
<th>P29mtP29</th>
<th>P29mtA11</th>
<th>A11mtP29</th>
<th>A11mtA11</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>**</td>
<td></td>
<td>*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>100</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>50</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**P<0.01, **P<0.001
Figure 3

A

**Figure 3A**

- **P29mtA11**
- 20 mM NAC treatment
- 20 μM Ebselen treatment

- **Counts**
- FL1-H

- **Legend**
  - Non-treated
  - 20 mM NAC, 24 h
  - Autofluorescence

B

**Figure 3B**

- **P29mtA11**
- 20 mM NAC treatment (days)
- 20 μM Ebselen treatment (days)

- **MCL-1**
- **β-Actin**

C

**Figure 3C**

- **Experimental metastatic potential (No. of lung nodules)**
- **Spontaneous metastatic potential (No. of lung nodules)**

- **P29mtA11**
- 20 mM NAC treatment (days)
- 20 μM Ebselen treatment (days)
- 10 mg/ml NAC administration

*Significant difference
**Very significant difference
Figure 4

A

B

C

Experimental metastatic potential (No. of lung nodules)