CD4+ T-Cell Response to Mitochondrial Cytochrome b in Human Melanoma

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Abstract
Mitochondrial DNA (mtDNA) is highly susceptible to mutations due to the low level of DNA repair and the presence of a high level of reactive oxygen species in the organelle. Although mtDNA mutations have been implicated in degenerative diseases, aging, and cancer, very little is known about the role of T cells in immunosurveillance for mtDNA aberrations. Here, we describe T-cell recognition of a peptide translated from an alternative open reading frame of the mitochondrial cytochrome b (cyt b) gene in melanoma cells established from a patient. To understand how the cyt b gene is transcribed and translated in tumor cells, we found that cyt b–specific CD4+ T cells only recognized protein fractions derived from cytoplasm and not from mitochondria. However, T-cell recognition of tumor cells could be inhibited by treatment of tumor cells with rhodamine 6G inhibitor, which depletes mitochondria. These findings suggest that cyt b mRNA is leaked out of the mitochondria and then translated in the cytoplasm for presentation to CD4+ T cells. The cyt b cDNAs from this patient contain highly heteroplasmic transition mutations compared with control cell lines, suggesting a compromise of mitochondrial integrity that may have contributed to melanoma induction or progression. These findings provide the first example of a mitochondrial immune target for CD4+ T cells and therefore have implications for the immunosurveillance of mitochondrial aberrations in cancer patients.

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Introduction
CD4+ helper T cells play important roles in orchestrating the host immune response against cancer and infectious diseases (1). Recent studies show that such cells are required for priming as well as secondary expansion of CD8+ memory T cells (2–4). They can also mediate tumor regression in the absence of CD8+ T cells, including MHC class II–negative or MHC class II–positive tumors (5, 6). Thus, the identification of MHC class II–restricted tumor antigens capable of stimulating CD4+ T cell responses has been critical for the development of effective cancer vaccines.

Over the past few years, a limited number of MHC class II–restricted tumor antigens have been identified in melanoma and other types of cancers (1) and have been classified into four major groups based on their expression patterns. The tissue-specific differentiation antigens, such as tyrosinase and glycoprotein 100, are expressed in melanoma as well as normal melanocytes. Tumor-specific shared antigens, such as LAGE-1 and NY-ESO-1, are expressed in many tumors but not in normal tissues, except testis. Antigens that are overexpressed in tumors include telomerase and MUC1. Tumor-specific unique or mutated antigens, such as CDC27 and LDLR-fucosyltransferase, result from point mutations and genetic alterations. These MHC class II–restricted and MHC class I–restricted tumor-associated antigens are derived from proteins localized in different subcellular compartments, including nucleus, cytosol, melanosomes, or plasma membrane (7–10). Although mitochondrial antigens, such as PDC-E2 (11, 12), aconitase (13), Nub0 (14), and ND1 (15), originated from nucleus and mitochondrion have been reported to be recognized by CD4+ and CD8+ T cells in various diseases, very little is known about mitochondrial antigens recognized by tumor-specific T cells. Because aberrant mitochondrial proteins are frequently observed in a variety of cancers, including melanoma (16), we postulate that some of these antigens may trigger an immune response.

Here, we describe the identification and characterization of a mitochondrially derived antigen, cytochrome b (cyt b), as a new MHC class II–restricted tumor antigen recognized by tumor-reactive CD4+ T cells. The T-cell epitope was derived from an alternative open reading frame (ORF) of the cyt b gene. These novel findings suggest a new role of CD4+ T cells in immunosurveillance for mitochondrial aberrations in cancer patients.

Materials and Methods

Reagents, cell lines, and cultures. Antibodies used in the histocompatibility leukocyte antigen (HLA)-blocking experiments were described previously (17). Anti-CD4 and anti-CD8 antibodies used for the fluorescence-activated cell sorting (FACS) analysis of CD4+ T cells were purchased from BD PharMingen (San Diego, CA). MHC class II pathway inhibitor chloroquine and mitochondrial inhibitor rhodamine 6G were purchased from Sigma-Aldrich (St. Louis, MO). Melanoma cell lines, EBV-transformed B-cell lines, and HEK293IMDR1 cells were established in our laboratory and maintained in RPMI 1640 (Biofluids, Gaithersburg, MD) supplemented with 10% FCS (Biofluids). CD4+ 1362 tumor-infiltrating lymphocytes (TIL) were cultured from a s.c. metastases resected from a patient (no. 1,362). T-cell lines were grown in RPMI 1640 containing 10% human AB serum (Valley Biochemicals, Winchester, VA) and 300 IU/mL interleukin 2 (IL-2; Chiron Corp., Emeryville, CA). T-cell sublines were generated by limiting dilution methods from a well plated at three cells per well as described previously (18).

cDNA library construction and screening. Total RNA was isolated from 1362mel cells using the RNaseasy system (Qiagen, Valencia, CA). Polyadenylated RNA [poly(A)+ RNA] was purified from total RNA by the PolyATtract system (Promega, Madison, WI). Approximately 5 μg poly(A)+ RNA was converted to cDNA using a cDNA construction kit (Life Technologies, Rockville, MD) with an oligo(dT) primer bearing a NotI site. Construction and screening of the cDNA library of 1362mel tumor cell RNA were similar to those described previously (10, 19). Plasmid DNAs

Note: K.S. Voo and G. Zeng contributed equally to this work.

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containing cyt b fragment were sequenced and assembled with SEQUENCER software (20).

Subcellular organelle fractionation, tumor cell lysate preparation, and peptide pulsing. EBV-transformed B cells were used as the antigen-presenting cells (APC) for the cell lysate and peptide-pulsing experiments. Subcellular fractionation of organelles from 586mel and 1362mel cells was done using sucrose gradient centrifugation approaches as described previously (21). Organelle lysate from fractionated organelles was obtained by repeated (thrice) freezing and thawing in the presence of 0.1% Triton X-100/PBS. Total cell lysate was prepared by repeated (thrice) freezing and thawing of tumor cells in PBS at \(-2 \times 10^7/\text{mL}\) followed by brief centrifugation at a low speed to pellet the unbroken cells and large cell debris. Protein concentration from individual fraction was determined by the bicinchoninic acid assay (Pierce Endogen, Rockford, IL), and an equal amount of protein was pulsed onto 1362 EBV-transformed B cells and incubated overnight. Pulsed EBV-transformed B cells were washed thrice before coculture with \(5 \times 10^4\) CD4+ T cells. Peptide pulsing experiments were pulsed onto EBV-transformed B cells at a various concentrations for 4 hours in RPMI 1640, washed twice with RPMI, and then cocultured with CD4+ T cells overnight for cytokine release assays.

Construction of cyt b-specific short hairpin RNA lentiviral vectors. Plasmid PLL3.7 was used to insert cyt b-specific short hairpin RNA (shRNA) oligos. The selection of coding sequences for shRNAs was empirically determined but started with an AG or AAG and analyzed by BLAST research to ensure that they did not have significant sequence homology to other genes in the database. The design and cloning of shRNAs oligos into PLL3.7 vector were done as described previously (22). The cyt b shRNA oligo consisted of 5'-TGCCGCAGAATCTCATATCT-3' (forward). FoxP3 shRNA served as a control. The FoxP3 shRNA oligo was 5'-GGCTTCATCTGTGGCATCA-3' (forward).

Results

Establishment of HLA-DR1-restricted tumor-specific 1362-D6 CD4+ T cells. Adoptive transfer of TILs together with a high dose of IL-2 has induced objective tumor regressions in melanoma patients (23). TIL.1362 cells were generated from a melanoma patient (no. 1362) who responded to a TIL plus high-dose IL-2 treatment in the Surgery Branch of National Cancer Institute (NCI; Bethesda, MD) and were found to recognize autologous tumor (Fig. 1A). To characterize these T cells further, we used limiting dilution methods to generate T-cell sublines that reacted strongly against autologous tumor cells (data not shown). One of the T-cell sublines, designated 1362-D6, was CD4+ based on FACS analysis (Fig. 1B) and was chosen for antibody-blocking experiments to determine its restriction elements. Recognition of autologous 1362mel cells by 1362-D6 T cells was specifically inhibited by monoclonal antibodies against MHC class II and HLA-DR molecules, but not by antibodies against HLA-DQ, HLA- DP, or MHC class I molecules (Fig. 1C), suggesting that these T cells recognize a tumor antigen presented by HLA-DR molecules.
Because 1362mel cells are positive for both HLA-DR1 and HLA-DR11 molecules, we sought to determine the specific HLA-DR restriction element for T-cell recognition. Tumor lysate was pulsed onto EBV-transformed B cells expressing only one of the HLA-DR molecules and used as target cells for T-cell recognition. HLA-DR1-positive 1363 EBV-transformed B cells were able to present antigen specifically from autologous tumor lysate to 1362-D6 CD4+ T cells, whereas autologous tumor lysate-pulsed HLA-mismatched EBV-transformed B cells or allogenic tumor lysate-pulsed HLA-matched 1363 EBV-transformed B cells failed to activate T cells (Fig. 1D). These findings suggest that HLA-DR1 is the restriction element for T-cell recognition and that the tumor antigen is unique to 1362mel cells. To confirm whether the tumor antigen is specifically presented by 1362mel cells, we tested a panel of HLA-DR1-expressing melanoma and EBV-transformed B-cell lines for T-cell recognition and found that 1362-D6 CD4+ T cells specifically recognized autologous 1362mel cells but not HLA-matched or mismatched tumor cells or autologous B cells (Fig. 1D), suggesting that 1362-D6 CD4+ T cells recognize a tumor antigen presented only by 1362mel cells.

Identification of cyt b as the gene encoding a tumor antigen recognized by 1362-D6 CD4+ T cells. To identify the gene that encodes the tumor antigen recognized by 1362-D6 CD4+ T cells, we used a genetic targeting expression system previously with MHC class II-restricted tumor antigens (7). Briefly, a cDNA library was generated from 1362mel RNA using 1362-D6 CD4+ T cells. cDNA clone 2 specifically stimulated 1362-D6 CD4+ T-cell responses, whereas control cDNAs failed to activate T cells (Fig. 2B). DNA sequencing analysis revealed that cDNA clone 2 contained a DNA fragment encoding the COOH-terminal end of mitochondrial cyt b protein, which is part of the respiratory enzyme complex III involved in electron transport (Fig. 2C).

Although cyt b is encoded by the mitochondrial genome and translated according to the mitochondrial code, ii and the fused cDNA are translated in the cytoplasm. By the nuclear genetic code, we found that the II leader sequence was fused to a 76-amino acid polypeptide translated from an alternative ORF of the cyt b gene. To determine the frequency of cyt b-specific T cells, we screened 40 T-cell sublines derived from TIL1362 against 293IMDR1 cells expressing an II-cyt b cDNA fragment and found that 4 of 40 T-cell sublines were capable of recognizing the cyt b antigen (Fig. 2D).

Identification of the cyt b epitope recognized by 1362-D6 CD4+ T cells. To identify the epitope from cyt b recognized by 1362-D6 CD4+ T cells, we initially made a series of peptides consisting of 15 to 19 amino acid with a HLA-DR1-binding motif. However, none of them were capable of activating T cells (data not shown). To refine the cyt b–specific T cells, we screened 40 T-cell sublines derived from TIL1362 against 293IMDR1 cells expressing an II-cyt b cDNA fragment and found that 4 of 40 T-cell sublines were capable of recognizing the cyt b antigen (Fig. 2D).

![Figure 2. Screening of an II-cDNA library constructed from 1362mel RNA using 1362-D6 CD4+ T cells. A, identification of a positive cDNA pool after screening II-cDNA library generated from 1362mel RNA. B, isolation of a positive cDNA clone encoding a tumor antigen recognized by 1362-D6 CD4+ T cells. 1362-D6 CD4+ T cells recognized 293IMDR1 cells transfected with the cDNA clone 2 but not with control cDNA or green fluorescent protein. C, schematic presentation of the nucleotide and amino acid sequences of the II-human mitochondrial cyt b fusion cDNAs. Protein residues are inferred from the third ORF of the cyt b gene using nuclear genetic codes. D, four 1362mel-specific CD4+ T-cell sublines recognized 293IMDR1 cells transfected with cDNA clone 2.](http://www.aacrjournals.org/canres/2006/66/11/figure_2.jpg)
showed that the 48-amino acid peptide could be readily detected by T cells at a peptide concentration of ~300 nmol/L (Fig. 3C). To further determine the minimal peptide for T-cell recognition, we did addition deletion analysis. Figure 3D shows that the shortest peptide capable of stimulating T cells is a 38-amino acid peptide, although further deletions led to loss of T-cell recognition. These results suggest that the 38-amino acid peptide derived from the translation of an alternative ORF of cyt b cDNA was presented to CD4+ 1362-D6 T cells.

**Cyt b is a tumor antigen recognized by CD4+ T cells.** To determine if cyt b is a true tumor antigen expressed naturally in 1362mel cells and recognized by 1362-D6 CD4+ T cells, we made a shRNA construct to knock down expression of the cyt b gene. This construct was cotransfected with Ii-cyt b cDNA into 293IMDR1 target cells and tested for its ability to inhibit 1362-D6 CD4+ T-cell recognition of the cyt b antigen. As shown in Fig. 4A, cyt b–specific shRNA molecules inhibited recognition of Ii-cyt b–expressing 293IMDR1 cells by 1362-D6 CD4+ T cells, in contrast to the control vector, which did not affect recognition. When autologous (1362mel) tumor cells were transduced with lentiviral constructs carrying cyt b shRNAs and tested against 1362-D6 CD4+ T cells, their recognition by the T cells was significantly inhibited (55%), whereas neither the control vector alone nor the FoxP3–specific shRNA (Fig. 4B) had any effect on recognition. To ensure that cyt b shRNA does not have off-target effects, we used CD4+ T cells with different antigen specificity. As shown in Fig. 4B, the 1359mel-specific CD4+ T-cell recognition of 1359mel target cells was not inhibited when the target cells were infected with a lentivirus-encoding cyt b shRNA, or FoxP3–specific shRNA was not inhibited. These results suggest that cyt b is a true tumor antigen expressed in 1362mel cells recognized by 1362-D6 CD4+ T cells.

**Cyt b transcript is derived from the mitochondrion.** Because the T-cell peptide was translated in the cytoplasm using nuclear genetic code, we sought to determine the cellular localization of the translated products. Figure 5A shows that protein fractions derived from tumor cell cytoplasm and lysosomes were capable of stimulating 1362-D6 CD4+ T cells when pulsed on HLA-DR1-expressing EBV-transformed B cells, whereas proteins fractions of mitochondria failed to stimulate T cells. These results suggest that cyt b T-cell peptides were translated and localized in the cytoplasm. We next sought to determine how cyt b mRNA was translocated to the cytoplasm of tumor cells. One possibility is that the cyt b gene was transferred and integrated into the nuclear genome. However, our Southern blot analysis using multiple restriction enzyme digests and tumor cell lines failed to identify a cyt b DNA fragment unique to 1362mel (data not shown), suggesting that cyt b gene in 1362mel might not be integrated into the nuclear genome of 1362mel cells. Alternatively, the cyt b transcript could be transcribed in the mitochondria but leaked out to the cytoplasm for translation. To test this possibility, we treated...
CD4+ T-cell recognition.

inhibitor specifically inhibited CD4+ T-cell recognition of processing. As shown in Fig. 5 inhibitor, a lysosomotropic agent that inhibits MHC class II antigen recognition of 1362mel cells in the presence of chloroquine.

mitochondrion is the source of nuclear tumor antigens were not affected, suggesting that CD4+ T cells that recognize mitochondrial-derived cyt b as a tumor antigen recognized by tumor cells with mitochondrion inhibitor rhodamine 6G for 2 days and then tested for T-cell recognition. Figure 5B shows that rhodamine 6G specifically inhibited 1362-D6 CD4+ T-cell recognition of 1362mel cells, whereas control CD4+ T cells that recognize nuclear tumor antigens were not affected, suggesting that mitochondrion is the source of cyt b transcript that encodes the T-cell peptide. To determine if the cyt b protein was processed using the MHC class II pathway, we tested 1362-D6 CD4+ T-cell recognition of 1362mel cells in the presence of chloroquine inhibitor, a lysosomotropic agent that inhibits MHC class II antigen processing. As shown in Fig. 5C, the addition of chloroquine inhibitor specifically inhibited 1362-D6 CD4+ T-cell recognition of 1362mel cells, whereas little inhibitory effects were observed with control CD8+ T cells that recognize MHC class I–restricted EBNA1 protein (24). Taken together, these results suggest that cyt b mRNA was leaked out of the mitochondrion for translation in the cytoplasm, which is then processed and presented to CD4+ T cells via the MHC class II antigen presentation pathway.

High percentage of mutations in 1362mel cells. Because 1362-D6 CD4+ T cells recognized 1362mel, but not HLA-DR1-matched tumor cells or EBV-transformed B cells, we reasoned that the cyt b gene in 1362mel tumor cells might contain mutations. To test this possibility, we sequenced >400 independent cyt b cDNA clones from 1362mel and HLA-DR1-matched melanoma cell lines (Table 1). Figure 5 shows extensive DNA mutations resulting in a change of cyt b residues specific for 1362mel cells, although only a few mutations were observed in autologous CD4+ T cells and other control melanoma cell lines. More than 50% of the cDNA clones sequenced contained mutations that were not seen in control cells and resulted in a 37% change in amino acid residues (Table 1). The majority of the mutations was heteroplasmic, whereas only three mutations were homoplasmic. One of the three homoplasmic mutations resulted in an amino acid change from L to I (residue 236) that was associated with ischemic cardiomyopathy (Fig. 6; ref. 25). Heteroplasmic missense mutants (5% of total cDNA clones) were also detected on W113R and W326R residues that were associated with exercise intolerance (26). A majority (81%) of the heteroplasmatic mutations were transition substitutions (T→C, 38% and A→G, 35%; Table 2), suggesting that T→C and A→G are common mutations in cyt b. This mutational spectrum is consistent with known hotspots from mitochondrial mutations commonly found in normal and tumor cells (27–30).

Discussion

This report describes for the first time the identification of mitochondrial-derived cyt b as a tumor antigen recognized by 1362-D6 CD4+ T cells. We showed its antigenic authenticity in 1362mel cells using gene knockdown experiments with cyt b–specific shRNA (Fig. 4B). We further showed by 5′-end and 3′-end deletion analysis that the epitope recognized by CD4+ T cells is derived from a 38-amino acid cyt b peptide (Fig. 3). However, the 38-amino acid peptide is not the final natural peptide presented on the tumor surfaces because it could not be presented to CD4+ T cells when pulsed onto HLA-DR1-expressing tumor cells or
293IMDR1 cells with poor endocytosis capability (data not shown). Further studies are needed to elucidate the mechanism by which natural cyt b peptides are generated for T-cell recognition. Our data favor the hypothesis that the cyt b mRNA is leaked out of the mitochondrion and then translated in the cytoplasm. We first showed that the cyt b peptide was derived from the cytoplasm and not from mitochondrion because protein lysate isolated from mitochondria was incapable of stimulating T cells when pulsed on APCs (Fig. 5A). This agrees with the II-cyt b cDNA that encodes the cyt b peptide using nuclear genetic code (Fig. 2C). However, we also showed that cyt b-specific T-cell recognition of autologous tumor cells was effectively inhibited by rhodamine 6G, which depletes mitochondrion (Fig. 5B), suggesting that mitochondrion is the source of cyt b transcript. These results are consistent with our Southern blot analysis using four restriction enzymes and four melanoma cell lines that failed to detect a cyt b DNA fragment unique to 1362mel cells (data not shown), suggesting that the cyt b gene is not transferred and integrated to the nucleus.

Mitochondrial cyt b is part of the respiratory enzyme complex III, which catalyzes electron transfer from succinate and NAD+-linked dehydrogenases to cytochrome c. Mutations in mitochondrial cyt b have been associated with several human diseases, including progressive exercise intolerance and complex III deficiency (31), myoglobinuria (32), Leber’s hereditary optic neuropathy (33), ischemic cardiomyopathy (25), parkinsonism/mitochondrial encephalopathy, lactate acidosis, and stroke-like episodes (34), and severe hypertrophic cardiomyopathy (35). All of these reported cases of cyt b mutations were heteroplasmic (~70-80% mutant genes in affected organs only), suggesting that they were somatic in origin. By contrast, the C→A mutation found at position 15452 in our patient’s cells, converting residue 236 of cyt b from leucine to isoleucine (25), was detected in 100% of the cyt b cDNA clones derived from 1362mel and 1362 T cells, suggesting a

Table 1. Summary of cyt b DNA and amino acid residue changes in cell lines

<table>
<thead>
<tr>
<th>Cell line, n (%)</th>
<th>1362mel</th>
<th>1362</th>
<th>1558mel</th>
<th>1558 EBV</th>
<th>1363mel</th>
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<tbody>
<tr>
<td>cDNA clone sequenced</td>
<td>192</td>
<td>80</td>
<td>96</td>
<td>48</td>
<td>96</td>
</tr>
<tr>
<td>Mutations</td>
<td>117 (60%)</td>
<td>9 (11%)</td>
<td>8 (8%)</td>
<td>4 (8%)</td>
<td>8 (8%)</td>
</tr>
<tr>
<td>Residue changes</td>
<td>71 (37%)</td>
<td>3 (4%)</td>
<td>4 (4%)</td>
<td>1 (3%)</td>
<td>3 (3%)</td>
</tr>
</tbody>
</table>

NOTE: Mutations were confirmed by sequencing from both DNA strands.

Figure 6. Identification of extensive mutations unique to 1362mel cells. Mitochondrial cyt b protein sequences of 200 cDNA clones derived from 1362mel cells were aligned with those obtained from autologous normal tissue (1362 CD4+ T cells) and HLA-DR1-expressing control melanoma cell lines (Table 1). Amino acid residues were inferred from the DNA sequences with the use of vertebrate mitochondrial codes. Protein sequences from the 1558mel cell line were used as a reference. Protein residue changes within each cell line were inferred from all of the cDNA clones sequenced. Mutant L236I, associated with ischemic cardiomyopathy, is denoted by "*" above the residue. Nonsense mutations, detected in a few cyt b cDNA clones from 1362mel and 1558mel cells, are indicated with an "x."
germ-line origin. Because patients carrying this mutation had a reduction in respiratory complex III activity (>50%), the mutation may play a role in the pathogenesis or progression of ischemic cardiomyopathy (25). The L236 residue, although highly conserved in many organisms, is located within transmembrane E, a region of the protein not associated with haem or quinone binding. Thus, the L236l mutation is unlikely to have an adverse effect on the bc1 complex (36), which may explain the prolonged survival of our patient whose mutation of this residue was homoplasmic.

Mitochondrial genome is useful for studies of human evolution, migration, and population histories because of its high copy number, maternal inheritance, lack of recombination, and a generally higher mutation rate than found in nuclear DNA (37). However, the accumulation of reactive oxygen species in some cells over time could induce a high level of mutations in both nuclear and mitochondrial DNAs (mtDNA), causing significant damage to mitochondrial and cellular proteins, lipids, and nucleic acids, which may predispose the host to cancer (38). Indeed, in our melanoma patient, ~37% of the cyt b protein in the mitochondria were mutated (Table 1), suggesting that these changes and perhaps others still to be identified in patient’s mtDNAs may contribute either directly or indirectly to tumorigenesis. Given that extensive mutations occurred in cyt b in tumor cells, we investigated whether mutations in cyt b contributed to recognition of cyt b antigen by T cells. We screened at least 20,000 cyt b cDNA derived from 1362mel using our 293IMDR1 cells as APCs. However, we failed to identify specific mutations responsible for T-cell recognition (data not shown). One possible explanation is that tumor cells may have the ability to more efficiently process and present T-cell epitopes than 293IMDR1 cells. Nonetheless, although our results indicated that the T-cell epitope encoded by a cyt b cDNA fragment did not contain mutations, mutations in other regions of cyt b may collectively contribute to aberrant expression of cyt b alternative gene products, which may directly or indirectly affect the immunogenicity of tumor.

Although cutaneous malignant melanoma has been convincingly linked with exposure to UV radiation (39, 40), few specific genetic mutations have been implicated in its induction and biological behavior (16). Our study provides the first example of the association of melanoma with cyt b mutations. Although the tumorigenic contribution of these mutations remains to be determined, it should be noted that an L236l homoplasmic mutation in a patient with decreased complex III activity is likely to increase the mitochondrial oxidative stress and activate nuclear DNA proto-oncogenes that could result in neoplastic transformation (41, 42). Most importantly, perhaps, our findings provide the first example of a mitochondrial immune target for CD4+ T cells and thus have potential implications for the immunosurveillance of mitochondrial aberrations associated with cancer.

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**References**

2. Janssen EM, Lemmens EE, Wolfe T, Christen U, von others still to be identified in patient’s mtDNAs may contribute

<table>
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<th>DNA substitution</th>
<th>No. mutations</th>
<th>Percentage of total</th>
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<tr>
<td>T → C</td>
<td>45</td>
<td>38</td>
</tr>
<tr>
<td>A → G</td>
<td>41</td>
<td>35</td>
</tr>
<tr>
<td>A → T</td>
<td>7</td>
<td>6</td>
</tr>
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<td>G → A</td>
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<td>A → C</td>
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<td>T → G</td>
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<td>2</td>
</tr>
<tr>
<td>C → A</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>A → T</td>
<td>8</td>
<td>6</td>
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NOTE: Number of mutation refers to total mutations obtained from all cDNA clones sequenced.


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