Published in final edited form as:

Leuk Res. 2009 November ; 33(11): 1499–1504. doi:10.1016/j.leukres.2009.03.012.

Identification of Mitochondrial Genome Concatemers in AIDS-associated lymphomas and lymphoid cell lines

Felipe Bedoya^{1,‡}, Maria M. Medveczky^{1,‡}, Troy C. Lund², Andras Perl³, Joseph Horvath⁴, Stephen D. Jett⁵, and Peter G. Medveczky^{1,*}

¹ Department of Molecular Medicine, College of Medicine, University of South Florida, Tampa, FL

² Department of Pediatrics, University of Minnesota, Minneapolis, MN

³ Department of Medicine, State University of New York, Syracuse, NY

⁴ Hemispherx Biopharma, Inc., New Brunswick, NJ

⁵ Department of Cell Biology and Physiology, University of New Mexico, Albuquerque, NM

Abstract

Since most oncogenic viruses persist as extrachromosomal covalently closed circular DNA (cccDNA) in tumor cells, we developed an assay to visualize and identify cccDNA in primary lymphomas. We identified concatemers of the mitochondrial genome in all samples analyzed, but not in normal lymphocytes. One AIDS-associated lymphoma (EL) was further studied in detail as its mitochondrial genome consisted of tandem head-to-tail duplications. Insertion of C-residues was noted near the origin of replication of EL mtDNA. EL cells responded weakly to Fas-apoptotic stimulus, displayed reduced mitochondrial activity and mass, and produced higher levels of reactive oxygen intermediates. Screening of several AIDS-associated lymphomas and established lymphoid cell lines also revealed the presence of mitochondrial genome concatemers consisting of interlinked monomer molecules. Taken together, our results suggest that formation of mtDNA concatemers is associated with oncogenic transformation in lymphoid cells.

Keywords

Mitochondrial DNA concatemers; AIDS-associated lymphomas; oncogenic transformation; leukemogenesis; mitochondria

1. Introduction

Non-Hodgkin's lymphoma is the most frequent type of cancer in AIDS patients [1–4]. Intriguingly, there are differences in lymphoma pathogenesis between transplant recipients and AIDS patients [1,4,5]. Most post-transplant lymphoproliferative disorders and lymphomas are

*Address correspondence to: Peter G. Medveczky MD, Department of Molecular Medicine, College of Medicine, University of South Florida, Tampa, FL 33612, USA. Fax: 813-396-9188; E-mail: pmedvecz@health.usf.edu.

‡These authors contributed equally to this work.

Conflict of interest statement

The authors declare no conflict of interest.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

due to proliferation of EBV-transformed B cells [6,7]. In AIDS-associated malignancies, EBV and KSHV have also been proposed as etiological agents, however, they have been associated with only 50% of Non-Hodgkin's lymphomas and no known viruses have been detected in the other half of the AIDS-associated lymphoma cases [2,3,5]. These observations suggest that half of AIDS-associated lymphomas may be induced by a not yet identified infectious oncogenic agent or triggered by a unique mechanism.

To detect putative unknown DNA virus episomes we screened primary AIDS-associated lymphomas. Surprisingly, these efforts led to the identification of concatemers of mitochondrial genomes in all lymphomas analyzed. It has been shown that mitochondrial genome mutations occur at high frequency in human tumors [9–13]. However, to date, mitochondrial genome concatemers have not been associated with any kind of cellular disorder. In this paper, we present the novel technique for the identification of mitochondrial genome concatemers and a detailed analysis of the mitochondrial genome in a unique case of AIDS-associated lymphoma.

2. Materials and Methods

2.1. Primary AIDS-associated lymphomas and T-cells

Twelve Non-Hodgkin's lymphoma samples from HIV-positive patients were obtained through the Division of Cancer Treatment and Diagnosis at the National Cancer Institute. Samples were cultured in RPMI 1640 medium supplemented with 4 mM L-glutamine, 10% fetal bovine serum and 50 µg/ml gentamycin. Peripheral blood mononuclear cells were isolated from blood bank donor samples and T-cell cultures were obtained by PHA-stimulation (10 µg/ml) of mononuclear cells for 3 days then cultured in medium containing 50U/ml of IL-2.

2.2. Method for detection of episomal DNA

Live samples of cells were selected by Lymphoprep™ (ProGen), according to manufacturer's protocol. 1×10^6 cells were loaded in each well of a vertical 0.8% agarose gel in 10% Ficoll buffer [8]. Cell lysis buffer containing 5% Ficoll, 1% SDS, 1mg/ml pronase and 0.05% xylene cyanol green was layered on top of the samples. Electrophoresis was at 0.8 V/cm for 3 h then 7.5 V/cm for 14 h at 4°C. The gel was stained with SYBRGreen® Nucleic Acid Stain (Lonza Rockland, Inc., Rockland, ME) for 1 hr, de-stained in 10mM Tris 1mM EDTA for 30 minutes and visualized using a Molecular Dynamics STORM Phosphor Imager (Model 860-PC, Amersham Biosciences, Piscataway, NJ). The DNA was vacuum-blotted onto a nitrocellulose membrane [14] and analyzed by Southern Blotting with human mtDNA-specific radiolabeled oligonucleotides (described below).

2.3. Purification of episomal DNA by alkaline lysis/CsCl-EtBr gradient

5×10^8 cells were centrifuged at 500xg for 15 min and resuspended in 10 ml 1X PBS. 5 ml of an alkaline buffer solution containing: 50 mM NaCl, 2mM EDTA, 1% SDS and 30 mM NaOH was added then vortexed for 2 min. Mixture was incubated at 30°C for 30 min then neutralized by 80.4 µl of 1M Tris pH 7.0 and 200 µl of 5M NaCl. Proteinase K (60 µg/ml) was added and incubated at 37°C for 30 min. DNA was phenol/chloroform extracted and ethanol precipitated. DNA pellet was resuspended in a 1X TE solution, CsCl 1.55 g/ml final density and ethidium bromide to 100 µg was added. The mixture was centrifuged at 45,000 RPM (Beckman L7–75, VTI65 rotor) for 72 hrs at 20°C. Fractions were collected and aliquots were analyzed by agarose gel electrophoresis.

2.4. PCR and sequence analyses

Purified cccDNA was electrophoresed in a low-melting temperature agarose gel ran overnight at 4°C, 22 volts. Gel fractions were melted 65°C for 10 min, phenol/chloroform extracted and ethanol precipitated. The following primers for amplification or as probes for Southern blotting were used [15]: 2F:5'CGATCAACCTCACCCTCT3'; 2R:5'TGGACAACCAGCTATCACCA3'; 7F:5'ACTAATTAATCCCCTGGCCC3'; 7R:5'CCTGGGGTGGGTTTTGTATG3'; 13F:5'TTTCCCCCTCTATTGATCCC3'; 13R:5'GTGGCCTTGGTATGTGCTTT3'; 17F:5'TCACTCTCACTGCCCAAGAA3'; 17R:5'GGAGAATGGGGGATAGGTGT3'; 22F:5'TGAAACTTCGGCTCACTCCT3'; 22R:5'AGCTTTGGGTGCTAATGGTG3'. Sequences of PCR products were determined and analyzed using CLC Workbench 3®.

2.5. Electron microscopy

Mitochondrial DNA samples were prepared for electron microscope imaging using a modified Kleinschmidt protocol [16,17]. DNA was mixed with ammonium acetate and cytochrome C, placed on a piece of parafilm to allow a protein monolayer to form. A carbon coated electron microscopy grid was touched the surface of the sample. Grids were washed by 95% ethanol/50 uM uranyl acetate. Grids were then either visualized as is or rotary metal shadowed with tungsten in an Edwards Auto 306 vacuum evaporator. The length of the DNA molecules was confirmed by comparison to plasmids of known length. The grids were imaged in a Hitachi H7500 TEM equipped with an Advanced Microscopy Techniques XR60 CCD camera. The NIH *Image J* was used to trace the backbone of the DNA molecules and the DNA length was calculated.

3. Results

3.1. Identification of novel cccDNA in AIDS-associated lymphoma

To visualize cccDNA the vertical gel electrophoresis method of Gardella *et al.* [8] was modified. Ten AIDS-lymphoma samples were screened by SYBRGreen™ staining (Fig. 1A) for large cccDNA. We observed DNA bands in some samples migrating between the loading well and linear broken DNA; this range of these gels contains large 15–200 kb cccDNA. Four AIDS-associated lymphoma bands were positive for EBV by Southern hybridization (not shown) and no longer studied.

To further study the six EBV- and KSHV-negative lymphomas we attempted to establish permanent cell lines. One lymphoma sample (EL) successfully yielded a cell line while all other EBV/KSHV negative lymphoma samples eventually died. Gel analysis of EL cells (Fig. 1B) and the derived cell line consistently demonstrated the presence of a large cccDNA.

To determine the sequence of the EL cccDNA, the cells were expanded in RPMI 1640 10% FCS without IL-2. CccDNA was isolated from EL cells by alkaline lysis followed by centrifugation on a CsCl-EtBr density gradient. Fractions of the gradient were collected and separated by low melting agarose gel electrophoresis. Figure 2A shows that the higher CsCl-density fractions corresponding to cccDNA contained two discrete bands and the lower density fraction contained smeared linear nuclear DNA as expected. DNA was extracted from the molten gel slice corresponding to the two cccDNA bands and digested with restriction endonuclease *MboI*. The restriction fragments were shotgun-cloned into the pBluescript™ vector (Stratagene) and the clones were analyzed by sequencing. BLAST results indicated that the clones contained 95% (>300 clones) human mtDNA and ~5% (~30 clones) human chromosomal DNA randomly derived from most human chromosomes, presumably caused by contamination from nuclear DNA during collection of fractions.

Then, normal T-cells were obtained from healthy blood donors and cccDNA was isolated and compared with EL DNA. Figure 2B shows that only one band was detected in normal T-cells while EL cccDNA contained two bands. Southern hybridization of this gel using a human-mtDNA probe shows co-migrating bands (slightly above the 23 kb marker) in both T-cell and EL DNA (Fig. 2C). A slower-migrating cccDNA from EL cells also hybridized with the mitochondrial probe (Fig. 2C). This supports our previous results that both forms of EL cccDNA were comprised of mtDNA. Moreover, the data show that EL cells contain some monomeric mtDNA and a larger and more abundant DNA hybridizing with the mtDNA probe.

3.2. The EL cccDNA is formed by tandem duplicated mitochondrial genomes

Electron microscopy analysis of 50 molecules from purified EL circular episomal DNA showed superhelical DNA with an average backbone contour length of 10.3 μm (Fig. 2D). Compared to the human mitochondrial genome (contour length of 5 μm) the cccDNA of EL cells consisted mainly of molecules equivalent to duplicated mtDNA.

To confirm that the EL episomal DNA is formed by duplicated tandem repeats, inverted repeats or rearranged mitochondrial genomes, full and partial digestion using BamHI (a single-cutter restriction enzyme) was performed. Figure 2E shows that complete digestion of EL cccDNA with BamHI yields one 16.5 kb band. Partial digestion yielded two linear bands of approximately 16.5 and 33 kb corresponding to putative monomers and dimers of mtDNA (Fig. 2F). These results confirm our electron microscopy and sequencing data that the EL episomal DNA is conformed by duplicated mitochondrial genomes organized in head-to-tail tandem orientation. Several additional restriction enzyme digestion data also support this conclusion (not shown).

3.3. Cytosine insertions are present in the control region of the EL mtDNA

To determine whether the EL mtDNA has suffered minor mutations, PCR amplification and DNA sequencing of purified EL cccDNA was done using specific mtDNA-derived oligonucleotides covering the entire mitochondrial genome (see methods). PCR products of EL and normal T-cell DNA were indistinguishable by agarose electrophoresis (not shown) indicating absence of large insertions or deletions.

The D-loop, which is the main regulatory region of mtDNA replication and transcription, has a highly-conserved C-stretch sequence (nt 311 to 315) known as the conserved sequence box II (CSBII) that has been reported as a mutational hotspot in primary tumors [18]. Figure 3 shows that sequencing of PCR-amplified EL mtDNA revealed two additional cytosine residues in the monomer and in the dimer within the CSBII near the primer of mtDNA replication.

3.4. Functional and structural mitochondrial properties of EL cells

Supplementary figure 1 summarizes various properties of EL cell mitochondria. In comparison with Jurkat cells (selected due to reproducibility throughout experiments and phenotypic similarity to normal T-cells, see reference 6 suppl. mat.), EL lymphoma cells showed increased ROI and peroxide-induced apoptosis as determined by staining with dihydroethidium (DHE) and AnnexinV-Alexa 647, respectively. These results suggest an unstable OXPHOS system in EL cells, since ROI could leak through uncoupled mitochondrial complexes (I-V), thus augmenting intrinsic pro-apoptotic signals. In contrast, monitoring of TMRM, NAO and AnnexinV-Alexa647 fluorescence indicated that mitochondrial membrane potential, mitochondrial mass and Fas-mediated apoptosis were respectively reduced in EL cells. Also, glutathione levels were significantly reduced in EL cells, which could explain the overproduction of ROI in these cells since glutathione works as a biological ROI scavenger.

3.5. Mitochondrial genome concatemers are present in primary AIDS-associated lymphomas and lymphoma cell lines

Primary AIDS-associated lymphomas and lymphoma cell lines were screened for episomal cccDNA using the Gardella technique (Fig. 4). Southern hybridization detected two bands in control T-cells; a broader and slower migrating circular form and a faster migrating linear form. However, all lymphoma cell lines contained an additional band co-migrating with EL cccDNA. Therefore, all lymphoma cells encode both monomer and concatemer forms of mitochondrial genomes. Small amounts of broken linear mtDNA were also present in all samples probably due to dying of some cells. The linear mitochondrial bands co-migrated with controls indicating lack of tandem duplication of mtDNA. In contrast, EL cells contained a unique larger and a faint smaller linear mitochondrial genome. The smaller was co-migrates with bands from all other cells confirming the presence of small amount of monomers in EL cells. Taken together, all lymphoma cell lines encode both mitochondrial genome monomers and concatemers consisting of two monomers probably due to interlinking of two unit-length mitochondrial circles.

4. Discussion

In this study we utilized a method developed in our laboratory for the visualization of cccDNA of mammalian cells. This technique requires only 1×10^6 cells and is ideal for screening large number of cell samples. It is rapid, reliable and can be concurrently harnessed for the detection of existing or novel episomes of viruses and for detection of size alterations such as insertions, deletions and concatemerization of mtDNA.

We identified mitochondrial genome concatemers in primary tumor suspension samples obtained from AIDS-associated lymphoma patients and in seven mammalian lymphoma cell lines. These concatemers were found to comprise of two kinds. All lymphoma cells, except EL cells, contained mitochondrial genome concatemers as interlinked monomers. In contrast, most EL mitochondrial genomes are comprised of two unit length molecules joined in head-to-tail configuration as demonstrated by several independent methods, such as Gardella gels, mtDNA partial digestion, and electron microscopy.

Although the molecular mechanism for mtDNA concatemer formation remains elusive, the observation that mtDNA can undergo homologous recombination in fungi [19], plants [20] and in humans between paternal and maternal mitochondrial genomes [21], represents an appealing model to examine within the lymphoma context. Malignant transformed cells have a higher rate of homologous recombination than normal cells [22] which could also explain mtDNA homoduplex and concatemer formation demonstrated in this work.

These experiments have also established a clear correlation between mtDNA concatemerization and malignancy of lymphoid cells. However, it is not clear whether concatemerization is a contributing factor to oncogenesis, or alternatively, a result of continuing growth of immortalized cells. Resolution of these possible alternatives requires further experiments.

Within the mitochondrial genome, a single major non-coding region the D-loop contains the main regulatory sequences for transcription and replication initiation. The mtDNA polymerase, DNAPol- γ , initiates replication at the origin bound with the replication primer, and proceeds along one DNA strand displacing the other strand [23]. In EL mitochondrial DNA, one to two cytosine insertions were present at a highly conserved sequence block within the D-loop, known as CSBII. Interestingly, insertion of C residues in the CSBII has been described as a frequent event in mtDNA alterations in human tumors [18].

EL cells have significant alterations in mitochondrial functions. Probably the decrease in Fas-mediated apoptosis is most noteworthy. It has been shown that tumor cells often escape immune surveillance by down-regulating the Fas pathway or by using it to their advantage [24]. For example, some tumors increase Fas ligand (FasL) expression to induce apoptosis of infiltrating lymphocytes [25]. Also, some viruses can promote their propagation by expressing Fas-inhibitory proteins [26,27]. In regards to mtDNA, the cleavage and cytoplasmic release of cytochrome b (a mtDNA-encoded protein) has been directly linked to Fas-induced apoptosis [24]. However, more studies are required to evaluate whether these changes in EL cells are a consequence of malignant transformation or represent the result of mtDNA duplication and mutation.

Significant reduction of glutathione levels in EL cells may be associated with the elevation in ROI production observed in these cells, since glutathione is a biological scavenger of ROI. Several lines of evidence have shown that high ROI levels are associated with cell survival by deregulation of the PI3-kinase/Akt pathway during tumorigenesis [28,29].

Azidothymidine (AZT), a thymidine analog that inhibits HIV reverse-transcription, has been one of the most widely used antiretroviral drugs in the treatment of AIDS. Due to substrate competitive inhibition, AZT has been shown as a DNAPol- γ inhibitor [30–32]. AZT treatment could result in the induction of impaired mtDNA replication that could promote the formation of mitochondrial genome concatemers and/or other mtDNA abnormalities, which may be associated to oncogenic transformation in individuals with AIDS. However, further investigation would be necessary to confirm this hypothesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Shara Pantry for helpful suggestions on this manuscript.

Abbreviations

MtDNA	Mitochondrial DNA
CSBII	Conserved sequence box II
cccDNA	Covalently closed circular DNA

References

1. Biggar RJ, Jaffe ES, Goedert JJ, Chaturvedi A, Pfeiffer R, Engels EA. Hodgkin lymphoma and immunodeficiency in persons with HIV/AIDS. *Blood* 2006;108(12):3786–3791. [PubMed: 16917006]
2. Carbone A, Gloghini A. AIDS-related lymphomas: from pathogenesis to pathology. *Br J Haematol* 2005;130(5):662–670. [PubMed: 16115121]
3. Grogg KL, Miller RF, Dogan A. HIV infection and lymphoma. *J Clin Pathol* 2007;60(12):1365–1372. [PubMed: 18042692]
4. Goedert JJ. The epidemiology of acquired immunodeficiency syndrome malignancies. *Semin Oncol* 2000;27(4):390–401. [PubMed: 10950365]

5. Knowles DM. Etiology and pathogenesis of AIDS-related non-Hodgkin's lymphoma. *Hematol Oncol Clin North Am* 2003;17(3):785–820. [PubMed: 12852656]
6. Loren AW, Porter DL, Stadtmauer EA, Tsai DE. Post-transplant lymphoproliferative disorder: a review. *Bone Marrow Transplant* 2003;31(3):145–155. [PubMed: 12621474]
7. Bellan C, Lazzi S, De Falco G, Nyongo A, Giordano A, Leoncini L. Burkitt's lymphoma: new insights into molecular pathogenesis. *J Clin Pathol* 2003;56(3):188–192. [PubMed: 12610094]
8. Gardella T, Medveczky P, Sairenji T, Mulder C. Detection of circular and linear herpesvirus DNA molecules in mammalian cells by gel electrophoresis. *J Virol* 1984;50(1):248–254. [PubMed: 6321792]
9. Brandon M, Baldi P, Wallace DC. Mitochondrial mutations in cancer. *Oncogene* 2006;25 (34):4647–4662. [PubMed: 16892079]
10. Gasparre G, Porcelli AM, Bonora E, Pennisi LF, Toller M, Iommarini L, Ghelli A, Moretti M, Betts CM, Martinelli GN, Ceroni AR, Curcio F, Carelli V, Rugolo M, Tallini G, Romeo G. Disruptive mitochondrial DNA mutations in complex I subunits are markers of oncogenic phenotype in thyroid tumors. *Proc Natl Acad Sci U S A* 2007;104(21):9001–9006. [PubMed: 17517629]
11. Mithani SK, Taube JM, Zhou S, Smith IM, Koch WM, Westra WH, Califano JA. Mitochondrial mutations are a late event in the progression of head and neck squamous cell cancer. *Clin Cancer Res* 2007;13(15):4331–4335. [PubMed: 17671113]
12. Petros JA, Baumann AK, Ruiz-Pesini E, Amin MB, Sun CQ, Hall J, Lim S, Issa MM, Flanders WD, Hosseini SH, Marshall FF, Wallace DC. mtDNA mutations increase tumorigenicity in prostate cancer. *Proc Natl Acad Sci U S A* 2005;102(3):719–724. [PubMed: 15647368]
13. Wallace DC. Mitochondria and cancer: Warburg addressed. *Cold Spring Harb Symp Quant Biol* 2005;70:363–374. [PubMed: 16869773]
14. Medveczky P, Chang C-W, Oste C, Mulder C. Rapid Vacuum Driven Transfer of DNA and RNA From Gels to Solid Supports. *Bio techniques* 1987;5(3):242.
15. Rieder MJ, Taylor SL, Tobe VO, Nickerson DA. Automating the identification of DNA variations using quality-based fluorescence re-sequencing: analysis of the human mitochondrial genome. *Nucleic Acids Res* 1998;26(4):967–973. [PubMed: 9461455]
16. Zahn RK, Tiesler E, Kleinschmidt AK, Lang D. A preservation and preparation process for desoxyribonucleic acids and their raw materials. *Biochem Z* 1962;336:281–298. [PubMed: 14003030]
17. Lang D, Mitani M. Simplified quantitative electron microscopy of biopolymers. *Biopolymers* 1970;9 (3):373–379. [PubMed: 5417647]
18. Sanchez-Cespedes M, Parrella P, Nomoto S, Cohen D, Xiao Y, Esteller M, Jeronimo C, Jordan RC, Nicol T, Koch WM, Schoenberg M, Mazzarelli P, Fazio VM, Sidransky D. Identification of a mononucleotide repeat as a major target for mitochondrial DNA alterations in human tumors. *Cancer Res* 2001;61(19):7015–7019. [PubMed: 11585726]
19. Bonnefoy N, Fox TD. Directed alteration of *Saccharomyces cerevisiae* mitochondrial DNA by biolistic transformation and homologous recombination. *Methods Mol Biol* 2007;372:153–166. [PubMed: 18314724]
20. Khazi FR, Edmondson AC, Nielsen BL. An *Arabidopsis* homologue of bacterial RecA that complements an *E. coli* recA deletion is targeted to plant mitochondria. *Mol Genet Genomics* 2003;269(4):454–463. [PubMed: 12768414]
21. Kraytsberg Y, Schwartz M, Brown TA, Ebralidse K, Kunz WS, Clayton DA, Vissing J, Khrapko K. Recombination of human mitochondrial DNA. *Science* 2004;304(5673):981. [PubMed: 15143273]
22. Thyagarajan B, McCormick-Graham M, Romero DP, Campbell C. Characterization of homologous DNA recombination activity in normal and immortal mammalian cells. *Nucleic Acids Res* 1996;24 (20):4084–4091. [PubMed: 8918816]
23. Clayton DA. Mitochondrial DNA replication: what we know. *IUBMB Life* 2003;55(4–5):213–217. [PubMed: 12880201]
24. Komarov AP, Rokhlin OW, Yu CA, Gudkov AV. Functional genetic screening reveals the role of mitochondrial cytochrome b as a mediator of FAS-induced apoptosis. *Proc Natl Acad Sci U S A* 2008;105(38):14453–14458. [PubMed: 18796602]

25. Krammer PH. CD95's deadly mission in the immune system. *Nature* 2000;407(6805):789–795. [PubMed: 11048730]
26. Thome M, Schneider P, Hofmann K, Fickenscher H, Meink E, Neipel F, Mattmann C, Burns K, Bodmer JL, Schroter M, Scaffidi C, Krammer PH, Peter ME, Tschopp J. Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. *Nature* 1997;386(6624):517–521. [PubMed: 9087414]
27. Bertin J, Armstrong RC, Otilie S, Martin DA, Wang Y, Banks S, Wang GH, Senkevich TG, Alnemri ES, Moss B, Lenardo MJ, Tomaselli KJ, Cohen JJ. Death effector domain-containing herpesvirus and poxvirus proteins inhibit both Fas- and TNFR1-induced apoptosis. *Proc Natl Acad Sci U S A* 1997;94(4):1172–1176. [PubMed: 9037025]
28. Clerkin JS, Naughton R, Quiney C, Cotter TG. Mechanisms of ROS modulated cell survival during carcinogenesis. *Cancer Lett* 2008;266(1):30–36. [PubMed: 18372105]
29. Galaris D, Skiada V, Barbouti A. Redox signaling and cancer: the role of “labile” iron. *Cancer Lett* 2008;266(1):21–29. [PubMed: 18374479]
30. Pinti M, Salomoni P, Cossarizza A. Anti-HIV drugs and the mitochondria. *Biochim Biophys Acta* 2006;1757(5–6):700–707. [PubMed: 16782042]
31. Martin JL, Brown CE, Matthews-Davis N, Reardon JE. Effects of antiviral nucleoside analogs on human DNA polymerases and mitochondrial DNA synthesis. *Antimicrob Agents Chemother* 1994;38(12):2743–2749. [PubMed: 7695256]
32. Yamanaka H, Gatanaga H, Kosalaraksa P, Matsuoka-Aizawa S, Takahashi T, Kimura S, Oka S. Novel mutation of human DNA polymerase gamma associated with mitochondrial toxicity induced by anti-HIV treatment. *J Infect Dis* 2007;195(10):1419–1425. [PubMed: 17436221]
33. Medveczky MM, Szomolanyi E, Hesselton R, DeGrand D, Geck P, Medveczky PG. Herpesvirus saimiri strains from three DNA subgroups have different oncogenic potentials in New Zealand white rabbits. *J Virol* 1989;63(9):3601–3611. [PubMed: 2547988]

Figure 1. Schematic representation of the Gardella technique and visualization of circular episomal DNA in AIDS-associated lymphoma EL cells

A) One million live cells in 10% Ficoll buffer are loaded into a well of a vertical agarose gel and digested by overlaying with a lysis buffer containing SDS and pronase and electrophoresis at 22 V for 3 hours. After electrophoresis at 70 volts for 18 h, the gel is stained and the DNA is visualized by staining with Sybr Green. The three different molecular configuration of DNA is indicated. **B)** SYBRGreen™ staining of Gardella gel of the unknown episomal DNA from EL cells (arrow) migrating within the range of large cccDNA.

	<i>Conserved Sequence Box II (CSBII)</i>	<i>Replication Primer</i>
<i>EL (C)</i>	ACCAAACCCCCCCC CC TCCCCCCCG	CTTCT
<i>EL (M)</i>	ACCAAACCCCCCCC CC TCCCCCCCG	CTTCT
<i>Normal T-cells (M)</i>	ACCAAACCCCCCCC--TCCCCCCCG	CTTCT
<i>Ref. Seq. (NCBI)</i>	ACCAAACCCCCCCC--TCCCCCCCG	CTTCT

Figure 3. Sequence alignment of conserved sequence box II (CSBII) regions from EL and normal mtDNA

A) Multiple DNA sequence alignments of EL cccDNA (mtDNA concatemers and monomers) and normal mtDNA were performed using *ClustalX*. Shown, the regions from the D-loop conserved sequence box II (CSBII, ranging nts 297–321) from *EL (C)*: EL mtDNA concatemer; *EL (M)*: EL mtDNA monomer; *normal T-cell (M)*: normal T-cell mtDNA and the human mtDNA reference sequence from NCBI.

Figure 4. Mitochondrial genome concatemers are present in primary lymphomas and lymphoid cell lines

Primary AIDS-associated lymphoma samples, various lymphoma cell lines and normal peripheral T-cell controls were analyzed by the method of Gardella *et al.* [8]. Southern blotting analysis was performed by using human mtDNA-specific radiolabeled primer probes (see methods). Samples: AIDS-associated lymphomas (2F7, 98-001, 98-002, EL); human body cavity lymphoma tumor cell line positive for KSHV (BCBL-1); B-cell Burkitt's lymphoma (Raji); *H. saimiri*-induced rabbit lymphoma (484Th) [33]; Non-Hodgkin's lymphoma positive for EBV and HIV (EBV-HIV-NHL).