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Elephant APOBEC3A cytidine deaminase induces massive double-stranded DNA breaks and apoptosis

Xiongxiong Li1,2, Vincent Caval1, Simon Wain-Hobson1 & Jean-Pierre Vartanian1

The incidence of developing cancer should increase with the body mass, yet is not the case, a conundrum referred to as Peto’s paradox. Elephants have a lower incidence of cancer suggesting that these animals have probably evolved different ways to protect themselves against the disease. The paradox is worth revisiting with the realization that most mammals encode an endogenous APOBEC3 cytidine deaminase capable of mutating single stranded DNA. Indeed, the mutagenic activity of some APOBEC3 enzymes has been shown to introduce somatic mutations into genomic DNA. These enzymes are now recognized as causal agent responsible for the accumulation of CG- > TA transitions and DNA breaks leading to chromosomal rearrangements in human cancer genomes. Here, we identified an elephant A3Z1 gene, related to human APOBEC3A and showed that it could efficiently deaminate cytidine, 5-methylcytidine and produce DNA breaks leading to massive apoptosis, similar to other mammalian APOBEC3A enzymes where body mass varies by up to four orders of magnitude. Consequently, it could be considered that eAZ1 might contribute to cancer in elephants in a manner similar to their proposed role in humans. If so, eAZ1 might be particularly well regulated to counter Peto’s paradox.

The APOBEC3 (A3) locus is bounded by two conserved genes, chromobox 6 and 7 (CBX6 and CBX7) in most placental mammals and encodes a family of cytidine deaminases capable of converting cytidine residues to uridine in single strand DNA (ssDNA). The mutagenic activity of these enzymes is involved with the restriction of retroviruses and DNA viruses, as well as endogenous retroelements and retrotransposons through hypermutation of viral DNA in a process called editing1. The A3 repertoire is extremely variable among mammals, the locus being shaped through extensive gene duplications and functionalization in the context of a virus-host arms race. A3 enzymes are made up of three related, but distinct zinc-finger domains referred to as Z1, Z2 and Z32–4 presumably already present in the genome of the ancestor of placental mammals5.

The last few years has seen the identification of two human endogenous A3 cytidine deaminases, APOBEC3A (A3A) and APOBEC3B (A3B) capable of introducing multiple mutations in chromosomal DNA6–9. These findings are grounded by the analysis of many cancer genomes, revealing far more mutations and rearrangements than hitherto imagined, where CG- > TA transitions appears to be the dominant mutations10–13. Human A3A is composed of a single Z1 domain, while A3B is composed of a double Z2Z1 domain, although only the C terminal Z1 domain being catalytically functional6. A3A and A3B enzymes are both localized in the nucleus and can edit cytidine residues to uridine in ssDNA during transcription and replication, following DNA repair, and leave TpC to TpT signature mutations that show up in cancer genomes6,8,9. Both enzymes can mutate 5-methylcytidine (5MeC) to thymidine leaving another distinct signature in cancer genomes6,14–16.

Although A3A and A3B are accepted as intrinsic mutators of cellular chromosomal DNA, analyzed in several cancer types8,11,17, debate still persists regarding the contribution of each enzyme in the accumulation of mutations paving the way for oncogenesis. While, it has been described that A3A and A3B could be enzymatically active in different cancers18, A3A is the more active of the two enzymes and as a consequence, only A3A can produce double stranded breaks (DSBs), at least in an experimental setting6,19. Editing frequencies of >0.5 can be found which is why the phenomenon is referred to as hyperediting or hypermutation1. Accumulation of substitutions localized in the A3B C-terminal domain attenuated the activity of the enzyme compared to A3A6.

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Interestingly, this functional attenuation was also observed for the rhesus monkey rhA3B enzyme compared to rhA3A indicating that this mutagenic dichotomy was maintained for ~38 million years. Moreover, the deletion of most of the A3B gene results in a higher odds ratio of developing breast, ovarian or liver cancer. Indeed, the A3B genes of most of the analyzed mammals are significantly divergent from their counterparts in other species, with the most notable exception being the rodent A3A gene. The rodent A3A gene is absent among all members of the order Rodentia, with the exception of the horse and cow. The deletion observed between human A3A and A3B genes was also observed in the A3B genes of other species, such as the dog and the elephant, which is consistent with the hypothesis that this deletion is a result of evolutionary divergence.

Another difference between A3A and A3B lies in their evolutionary history. A3A is present across most placental mammals, indicating that this evolutionary experiment has been running ~150 million years. There are some notable exceptions—an A3A gene is absent among all members of the order Rodentia, pigs, while for Felidae, the gene is inactive but identifiable. By contrast, A3B is unique to the order Primates and arose by gene conversion involving A3A. We have previously shown that the A3A enzymes from 8 mammalian species from rabbits to cows and horses were capable of deaminating C and 5MeC in ssDNA as well as producing DSBs, even though activities varied considerably.

The incidence of developing cancer was hypothesized to increase with the body size, referred to as Peto’s paradox. However, as large animals exist and do not invariably die of cancer this paradox fails to explain the presence of compensatory mechanisms that protect the genome. With this in mind, we were intrigued by a recent report showing that elephants appeared to have a lower-than-expected rate of cancer which might possibly be coupled to multiple copies of TP53 even though most were processed pseudogenes. It is equally possible that the A3 enzymes of large mammals could have been attenuated by mutation. Accordingly, we decided to explore the function of the elephant A3Z1 enzyme.

Results and Discussion

Synthesis and expression of elephant APOBEC3Z1 cytidine deaminase. To explore the implication of elephant A3 enzyme in tumorigenesis, in silico data mining was performed using blast/blat analyses of the genomic for A3Z1 like sequences. We retrieved an elephant A3Z1 sequence named like sequences. We retrieved an elephant A3Z1 sequence named eA3Z1, equivalent to the A3Z1 sequence named eA3Z1. A genomic for A3Z1 sequence named eA3Z1, equivalent to the A3Z1 sequence named eA3Z1, was validated by direct sequencing (Supplementary Figure S1) and identical to the previous BLAST/BLAT analysis search sequences from genomic data.

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Figure 1. Comparison of APOBEC3Z1 cytidine deaminases. CLUSTALW alignment of A3A proteins. Sequence conservation is depicted in red for each residue. Asterisks represent residues involved in zinc coordination responsible for enzymatic activity. Red asterisk represents the isoleucine amino-acid specific to Z1 domain. Structural motifs structures (α helix, β sheet and loop) are indicated.
Elephant APOBEC3Z1 editing of nuclear DNA and 5-methylcytidine. To assay catalytic activity, eA3Z1 and hA3A plasmids were transfected into HEK-293T cells and cellular lysates were used in a fluorescence resonance energy transfer assay based in vitro deamination assay where C to U conversion in a TAMRA-FAM-labeled DNA oligonucleotide allows fluorescence detection following cleavage by uracil-DNA glycosylase (UNG) activity. Elephant eA3Z1 activity was on a par with hA3A (Fig. 3a). To explore A3 hyperediting of chromosomal DNA, the HEK-293T-UGI cell line was transfected with eA3Z1 and hA3A plasmids. The HEK-293T-UGI cell line constitutively expresses uracil N-glycosylase (UNG) inhibitor (UGI) where UNG is the crucial enzyme involved in excising uracil from DNA. As UNG is rate limiting for the detection of hyperedited chromosomal DNA, inhibition by UGI is necessary. At 48 hours post-transfection, total DNA was extracted and TP53 DNA was amplified by 3D-PCR, a technique that selectively amplifies A3-edited ssDNA molecules. The lowest PCR denaturation temperature (Td) allowing amplification of unedited TP53 target DNA was 87°C (Fig. 3b). For eA3Z1 and hA3A transfections, 3D-PCR products were recovered at Tds as low as 84.1°C and 84.9°C respectively which is diagnostic for A3 editing (Fig. 3b). Nonetheless, 3D-PCR products from the 85.9°C amplification (white asterisk, Fig. 3b) were cloned and sequenced as those of the last positive amplification for plasmid vector (pv) and hA3A C101S as negative controls. Hyperedited TP53 target sequences were recovered with an average editing frequency of 10% compared to a background value of 0.6% (Fig. 3c). The monotony of editing is confirmed by the frequency of non-CG- > TA mutations which did not differ from background values. Cytidine editing was strongly associated with TpC, and to a lesser extent CpC dinucleotides (Fig. 3d) to the detriment of GpC and ApC which is typical for mammalian A3A enzymes.

To demonstrate that no endogenous activity of hA3A present in HEK-293T-UGI cells would give rise to hypermutated sequences, hA3A or eA3Z1 transfections were performed in QT6 cell lines in presence of UGI. QT6 is a quail cell line and was chosen as there is no endogenous A3 background. As expected, 3D-PCR product sequence analysis demonstrated the same profile of hypermutated sequences and dinucleotide contexts (data not shown).
One of the singular traits of mammalian A3 deaminases is their ability to efficiently deaminate 5MeC. To demonstrate that eA3Z1 can deaminate 5MeC, QT6 cells were transfected with the eA3Z1 expression plasmid and subsequently transfected by 5MeC-substituted HIV env DNA fragments. As shown in Fig. 4a, 3D-PCR products were recovered at temperatures as low as 75.7°C and 77°C for the eA3Z1 and hA3A transfections respectively, compared to 82.1°C for the plasmid vector or hA3A C101S (Fig. 4a). The 82.1°C and 80.3°C 3D-PCR products (Fig. 4a), indicated by an asterisk were cloned, sequenced and confirmed the presence of edited 5MeC in the expected TpC dinucleotide context (Fig. 4b,c). Hyperedited 5MeC V1V2 target sequences were recovered with an average editing frequency of 5% and 6.8% respectively for hA3A and eA3Z1 compared to background value (Fig. 4c).

Elephant APOBEC3Z1 induces double strand DNA breaks and apoptosis. Human A3A editing of chromosomal DNA results in the formation of DSBs and can be readily scored by analysis of histone variant H2AX phosphorylation at serine 139 (γH2AX), a well-known marker for DSBs and the DNA damage response. HeLa and QT6 cell lines were transfected with eA3Z1 and hA3A constructs ± UGI using plasmid vector as negative control. As can be seen in Fig. 5a, eA3Z1 and hA3A generated DSBs in HeLa cells ∼35 and ∼20-fold over background. While DSBs were more pronounced in QT6 with ∼70 and ∼40-fold higher over plasmid control with eA3Z1 being the more active of the two enzymes (Fig. 5b). In the presence of UGI, a decrease in A3-induced DSBs was noted for hA3A and eA3Z1 transfected cells indicating that UNG plays an important role in the formation of DSBs upon DNA editing. As A3-induced DSBs lead to apoptosis, we measured cytochrome c release (Fig. 5c). Transfection of equal amounts of plasmid DNA resulted in greater cytochrome c release for eA3Z1 using Annexin V and propidium iodide as markers for apoptosis (Fig. 5c,d). These data demonstrate that eA3Z1 is a strong editor of ssDNA and can induce DSB leading to apoptosis.

Taken together, these data demonstrate that eA3Z1 clearly exhibit an enzymatic activity similar to human and other mammalian A3A cytidine deaminases since eA3Z1 edits both C and 5MeC residues in ssDNA and can make DSBs leading to apoptosis. A side-by-side comparison of eA3Z1 to 8 mammalian A3As is shown in Fig. 5e.
constructs are well expressed by Western blotting and immunofluorescence with the signal exception of the tamarin construct 26. While eA3Z1 is well ranked among the series, it is less efficient than the cow and horse constructs which, although they have large body mass and longevity (wild cattle 18–25 + years, up to 900 kg; horses 30–40 years, up to 600 kg), are not comparable to the elephant (median 56 years, up to 70 years, up to 7000 kg). Obviously, there are many variables that can alter A3 function: mi- and lncRNAs and transcription factor sites in the promoter as well as the role of negative interactors like TRIB334,40 which is part of the broad CtIP-Rb-BRCA1-ATM protein network that involves cell cycle control, cell survival, DNA repair, and genome stability. The eA3Z1 described here is clearly comparable to those of many large mammals, being able to damage chromosomal DNA and might therefore contribute to oncogenesis. If so, perhaps the A3s of elephants must be tightly regulated to lower the incidence of cancer.

Methods

Plasmids and samples. Elephant A3Z1 cDNAs were synthetized (GeneCust) and subsequently cloned into pcDNA3.1/V5-His-TOPO vector (Invitrogen). All constructs were C-tagged by the V5 epitope. Catalytically inactive hA3A mutants, UGI expression plasmid and other mammalian A3A expression plasmids, sheep, tamarin, rabbit, rhesus monkeys, dog, cow and horse were already described. All plasmids were verified by sequencing. A fatal case of encephalomyocarditis virus involving an African elephant (Loxodonta africana) occurred in November 2013 at the Réserve Africaine de Sigean in France 32. Naturally infected samples were collected as part of routine veterinary investigation carried out by qualified veterinarians in the area of origin. All methods were carried out in accordance with relevant guidelines and regulations.

Cell transfection. Approximately 800,000 HeLa, HEK-293T, HEK-293T-UGI and QT6 cells were seeded into 6-well plates and transfected with 2 µg of A3 plasmid using the jetPRIME transfection kit (Polypus Transfection™) according to manufacturer’s instructions. For cotransfections, a plasmid ratio of 1:1 was used.

Deamination assay. At 48 hours post-transfection, A3-transfected 293 T cells were extensively washed with PBS and mechanically harvested. Total proteins were extracted using specific lysis buffer (25 mM HEPES pH 7.4, 10% glycerol, 150 mM NaCl, 0.5% Triton X-100, 1 mM EDTA, 1 mM MgCl2, 1 mM ZnCl2) supplemented with

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**Figure 4.** Elephant APOBEC3Z1 deamination of 5-methylcytidine. (a) 3D-PCR recovered edited 5MeC substituted HIV env DNA down to 77°C and 75.7°C for hA3A and eA3Z1 respectively. The white line indicates the threshold between edited and unedited 3D-PCR products in terms of the denaturation temperature. Pv and hA3AC101S showed no editing of 5MeC substituted HIV env DNA and were used as negative control. Asterisks refer to the samples cloned and sequenced. M, molecular weight markers. (b) Dinucleotide context for human and elephant A3A deamination of 5MeC. Chi-square test indicates dinucleotide frequencies that significantly deviate from the expected values (‘p < 0.05). (c) CG- > TA mutation frequencies analyzed with hA3AC101S and pv at 82.1°C and with hA3A and eA3Z1 at 80.3°C.
protease inhibitors. Deaminase activity was assessed by incubating whole cell lysates with 1 pmole DNA oligonucleotide 5′FAM-AAATTCTAATAGAT AATGTGA-TAMRA in the presence of 0.4 unit of uracil-DNA-glycosylase (UDG) (New England Biolabs) in a 20 mM Tris-HCl, 1 mM dithiothreitol, and 1 mM EDTA reaction buffer. After 2 hours of incubation at 37 °C, abasic sites were cleaved by heating for 2 min at 95 °C and end point fluorescence was measured using a RealPlex2 Mastercycler (Bio-Rad) with FAM setting and background fluorescence obtained with mock-transfected cells as negative control. Results are normalized to the quantity of protein using Pierce BCA protein assay kit (Thermo Scientific).

RNA and DNA extraction, 3D-PCR amplification and cloning. Total RNA from the elephant liver and DNA from transfected cells were extracted using the MasterPure™ complete DNA and RNA purification kit (Epicentre) and suspended in 35 µL of sterile water. cDNAs were synthesized using QuantiTect reverse transcription kit (Qiagen). For eA3Z1 amplification, a semi nested PCR was performed. For PCR1 and PCR2, first round primers were 1MYfwd: 5′CTGATGGATCAAAACATATTCCGCTTCA and 2MYrvt: 5′CTGATGGATCAAAACATATTCCGCTTCA and 1MYrvt: 5′TGGGCACAGTTACGGCAGGGACTC. And for PCR2, second round primers were: 2MYfwd: 5′GGCCGAAACAGACCTACCTACCTTGC and 2MYrvt: 5′CTGATGTGTTTCCATTGTGAGAATAC. First and second round reaction parameters were 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 sec., 60 °C for 30 sec. and 72 °C for 1 min., with final extension for 10 min. at
72°C. PCR1 and 2 products direct sequencing were outsourced to Eurofins and performed using amplification primers: 1MYfwd, 1MYrev, 2MYfwd and 2MYrev (accession number: MK136802).

For TP53 amplification, primers were: TP53outf 5′GAGCTGGACCTTAGCTCCAGAAAGGACAA TP53outrev 5′GCTGGTGTTGCAGGGGCAGTGCAGGAA, amplification was performed using first-round standard PCR with 5 μL of DNA extract followed by nested 3D-PCR8,9 with 5 μL of 1/50 dilution of the first PCR round. Primers for nested 3D-PCR were: TP53in 5′TTCTTTTTCCATCTCGAGTGGTAA and TP53inrevr 5′AAAGTGGATAAAAAGTGAATGCAGGCTAA, 3D-PCR was performed in 50 μL with 1 U Taq DNA polymerase (Eurobio) per reaction. PCR conditions for the first round of amplification were 5 min. of denaturation at 95°C then 40 cycles of amplification (30 sec. 95°C, 30 sec. 58°C, 30 sec. 72°C), followed by 7 min. at 72°C. The condition of 3D-PCR were 5 min. of denaturation temperature gradient at 94–82°C for 40 cycles of amplification (1 min. 84–92°C, 30 sec. 58°C, 30 sec. 72°C, followed by 10 min. at 72°C.

For the 5MeC deamination assay, a 679 bp fragment of HIV-I Lai env gene was amplified using total substitution of dCTP by 5Me-dCTP (Trilink) using the primer pair MC1, 5′TTGATGCTCTGTGGCTACAGCA and MC2, 5′GCCCTATTTCCATGTGACATTGTA. The 5MeC containing DNA was heat denatured and chilled on ice and 200 ng of synthesized DNA was transfected using jetPRIME 24 hours following initial transfection of A3 coding plasmids in QT6 cells as described earlier.5 The second round PCR was classical PCR, primers were: MC3 5′TGTTCAACAGACCCACACACCAAA and MC4 5′TCCATTGGAAGCTCCTATTATTACA, and PCR parameters were: 95°C for 5 min., followed by 30 cycles of amplification (45 sec. 95°C, 45 sec. 54°C, 90 sec. 72°C) followed by 20 min. at 72°C. The 3D-PCR reaction parameters were 75–88°C for 5 min., followed by 35 cycles of amplification (45 sec. 75–88°C, 45 sec. 56°C, 90 sec. 72°C) followed by 20 min. at 72°C. For monitoring of (45 sec. 75–88°C, 45 sec. 56°C, 90 sec. 72°C) followed by 20 min. at 72°C. For monitoring of (45 sec. 75–88°C, 45 sec. 56°C, 90 sec. 72°C) followed by 20 min. at 72°C. For monitoring of 3D-PCR products were cloned into the pCR2.1 TOPO cloning vector (Invitrogen) and sequenced (Eurofins).

Immunofluorescence. Approximately 50,000 HeLa cells were seeded in Nunc™ Lab-Tek™ II Chamber Slide™ System Thermo Scientific™ and transfected 24 hours later with 1 μg of plasmid DNA according to the Fugene® protocol. Two days after transfection, coverslip grown transfected HeLa cells were washed three times with PBS and fixed with 4% paraformaldehyde (Electron Microscopy Sciences) for 15 min. Cells were then washed two times and permeabilized with a 50% methanol/aceton mix for 10 min. After two PBS washings, permeabilized cells were incubated for 1 hour at room temperature, first with 0.5% bovine serum albumin (BSA) PBS 1/200 mouse monoclonal anti-V5 antibody (Invitrogen) and then with 0.5% bovine serum albumin PBS 1/500 anti mouse Alexa Fluor 488 conjugated antibody (ThermoFisher). After several PBS washings, coverslips were mounted with Vectashield mounting medium for immunofluorescence (Interchim). Imaging was performed using a Leica SP5 confocal microscope. 

FACS analysis, double strand-breaks and apoptosis. Transfected cells were trypsinized, washed with PBS, fixed in 2% ice-cold paraformaldehyde (Electron Microscopy Sciences) for 10–20 min. on ice. After one PBS washing, cells were permeabilized in 90% ice-cold methanol (Sigma) for 30 min. For DSBs experiments, fixed and permeabilized cells were incubated 1 hour on ice with 1:200 PBS-0.5% BSA diluted mouse monoclonal anti-V5-Tag Alexa Fluor® 488 antibody (AbD Serotec) and 1:100 diluted Alexa Fluor® 647 Mouse anti-H2AX (BD Pharmingen). For apoptosis, transfected HeLa cells were collected and washed with PBS, then incubated with complete DMEM medium at 37°C for 2 hours. After washing with cold PBS, cells were resuspended in 1X Binding Buffer (BD Pharmingen) and then counterstained with 1 μg/ml FITC Annexin V antibody (BD Pharmingen) and 5 μg/ml Propidium Iodide (PI) (BD Pharmingen) to distinguish between early apoptotic and late apoptotic necrotic events. Treatment by 100 mM etoposide in dimethylsulfoxide was used as positive control. The labelled samples were analyzed on a MACSQuant® analyzer harboring violet, blue, and either a red laser (measure of dsDNA breaks and apoptosis). The data were analyzed using the FlowJo® software (Tree Star Inc., version 10.1r5 for Mac).

Mitochondrial cytochrome c release. At 48 hours post-transfection, HeLa cells were trypsinized and investigated for cytochrome c release by using the FlowCellect cytochrome c kit from Millipore according to manufacturer's instructions. Cells treated with 200 μM etoposide for 16 hours were used as a positive control of cytochrome c release. Stained samples were acquired on a MACSQuant Analyzer (Miltenyi Biotec) and the data were analyzed with FlowJo software (Tree Star Inc. version 10.0.8). For each sample 10,000 cells were counted.

Western blotting. Cells were recovered 48 hours after transfection. Protein extraction and Western blot analysis were carried out according to standard procedures. After blocking, membranes were probed with either a 1:5000 dilution of anti V5-tag horseradish peroxidase-coupled antibody (Invitrogen), or a 1:15000 dilution of anti β-actin (Sigma). The membrane was subjected to detection by SuperSignal™ West Pico chemiluminescent substrate (ThermoFisher Scientific).

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Author Contributions
X.X.L. and V.C. performed the experiments and analyzed the data. S.W.H. and J.P.V. designed the experiments, analyzed the data and wrote the manuscript.

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