A transforming giant virus discovered in Canine Transmissible Venereal Tumour: Stray dogs and Tasmanian devils opening the door to a preventive cancer vaccine.

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ABSTRACT

Background: Canine Transmissible Venereal Tumour (CTVT) along with Tasmanian Devil Facial Tumour and transmissible leukaemia in Mya Arenaria soft shell-clams are the only examples of contagious cancers occurring in nature. In particular, CTVT is the oldest contagious cancer present in the wild world, emerged 11,000 years ago.

The attempts to detect a transmissible virus as a causative agent in these form of contagious cancer proved conflicting and the current consensus view is that a transformed cell itself is transmitted and starts the tumor in a new animal, as a parasitic allograft. We modify this perception and report for the first time the isolation of an acutely transforming microbial infectious agent from CTVT.

Methods: Giant viral particles were successfully isolated from CTVT specimens through a sucrose gradient, examined at electron microscopy, fully sequenced, used for transformation tests on NIH-3T3 cells and tumorigenic experiments in dogs.

Results: The particles isolated from CTVT are giant viral particles with 1.17MB mega-genome. They transformed NIH-3T3 cells in vitro and initiated the typical CTVT lesions in a healthy dog, just one week post-infection. Only the fraction containing the giant viral particles were able to reproduce the tumour, while a filtered supernatant did not. This ruled out the presence of classic filterable viruses.

Conclusions: We discovered an infectious microbial agent, acutely transforming in CTVT. The agent shares some of the features that apply to giant Mimiviruses, such as the gigantic size and the presence of a mega-genome. However, distinct from environmental giant Mimiviruses, this mammalian microbial entity has a transforming nature and does not require amoeba co-culture. The biological characteristics depict this large agent halfway in between a classic oncogenic virus and a self-governing microbial cell-like entity.

A practical application of this finding could be a new vaccination plan to control the spreading of this contagious cancer among dogs in endemic area as well as a new approach to save the Tasmanian devils that share the same cancer's tale with stray dogs.

Significance Statement

The discovery of this infectious large agent in CTVT in dogs not only modifies our current beliefs on the origin of transmissible cancer in the wild, but offers alternative anti-cancer strategies that aim to eradicate an entire microbe and its mega-genome, carrying hundreds of proteins. Unlike conventional oncoviruses with a small genome and one or two transforming proteins, these transforming microbial entities are more similar to a self-governing cell, a sort of cell–like entity specialized in carcinogenesis, halfway in between a classic oncogenic virus and an eukaryotic cancer cell.

The awareness, resulting from this finding, that contagious cancers may be more widespread than previously thought, opens the door to a preventive cancer vaccine in humans and in the wild, leading to the development of strategies to target an entire shuttling system of oncogenes, all enclosed in a single infectious microbe, and not just a solitary protein involved in carcinogenesis.

Introduction

Three transmissible cancers are known to have emerged naturally in the wild: canine transmissible venereal tumour (CTVT), Tasmanian devil facial tumour disease (DFTD) and a leukaemia-like cancer in Mya Arenaria soft-shell clams. These cancers are contagious and spread between individuals (1-2).

CTVT, also known as Sticker's sarcoma, is the oldest cancer known in nature and first emerged in a dog that lived about 11,000 years ago (3-4). It is a venereal transmissible sarcoma that was initially described by Novinsky in 1876, who demonstrated that the tumour could be transplanted from one susceptible host to another by inoculating it with cancer cells (5). Some authors suggested a viral agent in the aetiology of this cancer (6), however, the tumour could not be transmitted by cell free extracts (7). Therefore, the current consensus view is that a cancer cell itself or cluster of cancer cells are transferred between individuals without the involvement of an infectious agent. According to this paradigm, the transformed cells of the neoplasm are the vectors of transmission, implanting onto genital mucosa during mating (8).

Devil facial tumour diseases (DFTD) is a recent transmissible cancer that emerged in Tasmania approximately 20 years ago and since then, the cancer has decimated the Tasmanian devil population (9-10). DFTD cancer, appears as large solid ulcerated masses destroying animals face and mouth. No protective immunity has been established and every infected animal dies from cancer. Since the Tasmanian devils only exist in Tasmania, the cancer has the potential to cause the extinction of the species. Like the CTVT, it is proposed that the tumour is transmitted by an initial cancerous cell, transmitted as an allograft through animals' bites (11-13). However, the mechanisms used by DFTD tumour cells to escape the immune system in its allogenic hosts are not completely understood.

Dogs and Tasmanian devils offer the only current examples of transmissible tumours among mammals. However, to fully explore the biology of transmissible tumours, it is essential to consider also non-mammalian species. *Mya Arenaria* soft shell clam, since the late 1960's, has been the subject of numerous reports of a disseminated leukaemia-like neoplasia. The strongest evidence that this form of clams' neoplasia is transmissible comes from studies of unfiltered hemolymph that induce cancer in healthy individuals (14-15). Subsequent studies were consistent with a retrovirus aetiology where the examination of the hemolymphatic transcriptome identified the presence of retroviral protease, RT and integrase (15-17). Surprisingly, the highest RT levels was detected in cultured hemocytes compared to cell-free extracts (18). However, once again, the failure to detect any infectious agent in the clams deposed that the cancer derives from a single cell that became a cancerous cell.

The evidences that only unfiltered tumour tissue could transmit cancer in healthy animals and the failure to identify etiological viral agents in filtered cell-free extracts, made us wonder if the current epistemological definition of viruses and the common filtration techniques missed giant microbial agents in transmissible cancer. In fact, classical virus isolation procedures include filtration through 0.2-µm-pore filters to remove bacteria and protists. However, these filters also often exclude large microbes. The discovery of giant Mimiviruses infecting amoeba highlighted how ad example the use of a 0.45 µm filter may prevent the recovery of giant viruses (19-24). Therefore, following the same protocols, currently in use for the isolation of amoeba giant viruses, we explored the possible presence of giant microbial entities in clonally transmissible cancer, but in the isolation process, we did not use amoeba co-culture.

In this paper, we report the discovery of a transmissible giant microbial agent, acutely transforming, successfully isolated from CTVT. This finding modifies the current paradigm on contagious cancer in nature and offers new scenarios and anti-cancer strategies.

Results

Electron Microscopy of giant viral particles isolated from CVTV

CTVT penile tumour biopsy from two stray male dogs was homogenized and giant viral particles were isolated after ultracentrifugation on sucrose gradient (Figure 1). A white band was recovered at 25-30% interface -the same sedimentation rate of giant viruses infecting amoeba. However, these mammalian oncogenic giant viruses are distinct from amoeba giant viruses since they manifest a different tropism and do not require for their propagation amoeba co-culture. An in depth EM morphological analysis of the purified giant viral particles revealed the presence of an ancestral system composed of a large microbial particle and smaller satellite giant viral particles.

The large unicellular particle, that resembles an unicellular organism, has an average size of 600 -700 nm**,** but can reach up to 1 μm (Figures 1-2). The satellite particles are giant viral particles, Mimiviruses-sized (Figure 3). In some micrographs, the satellite giant viral particles can be seen trafficking inside the large unicellular organism, suggesting an interconnection between the large unicellular particle and satellite giant viral particles (Figure 4).

A morphological comparative study shows that these canine microbial entities have the same morphology of analogous giant viruses isolated from human T cell leukaemia (25-27), disclosing the same microbial cluster of a large unicellular organism and satellite giant viral particles (Figure 5).

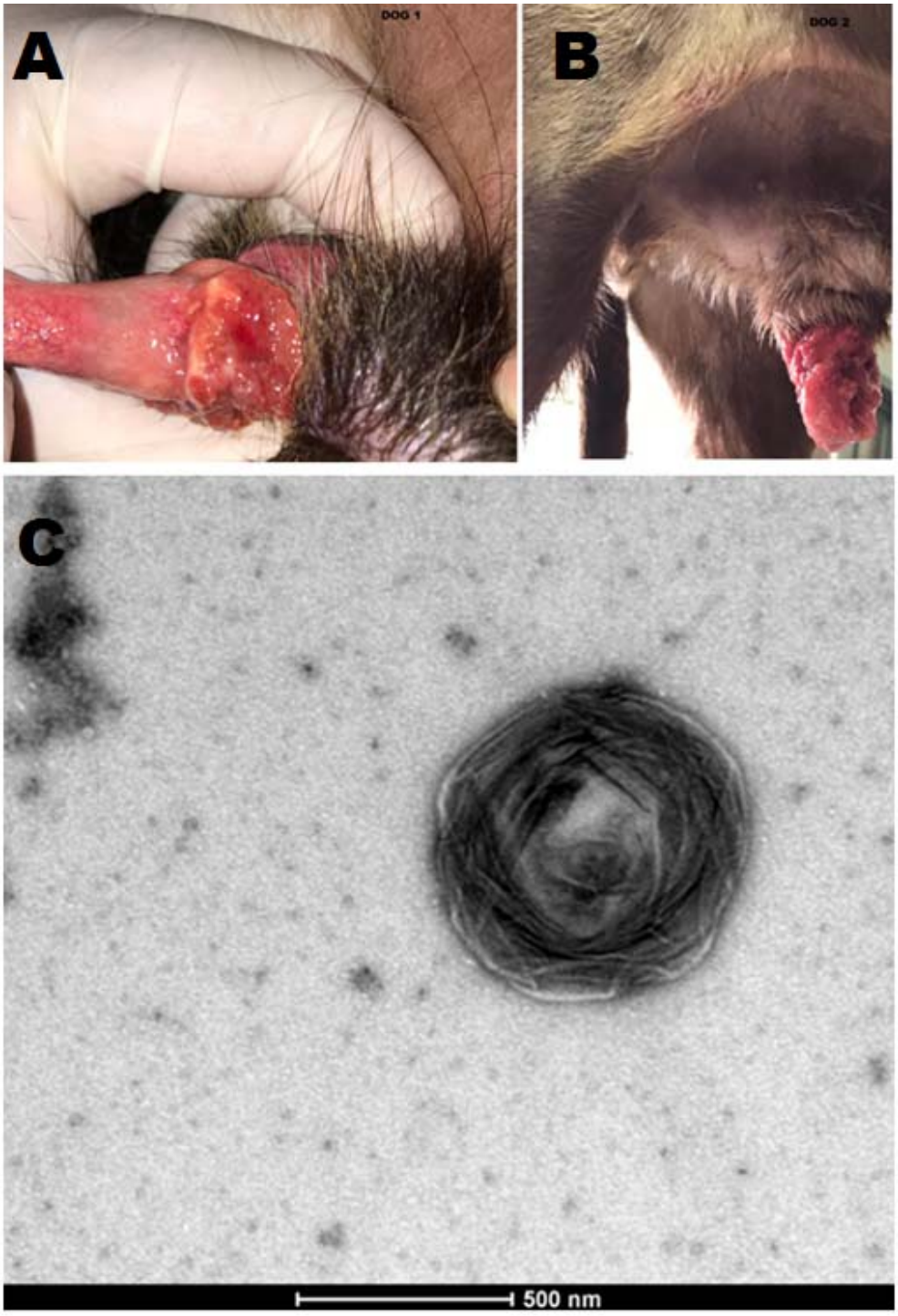

Figure 1. A-B) Penile CTVT from two affected male dogs. C) Detailed EM of a giant microbial particle isolated from CTVT. This large particle resembles unicellular organisms and has an average size of 700 nm.

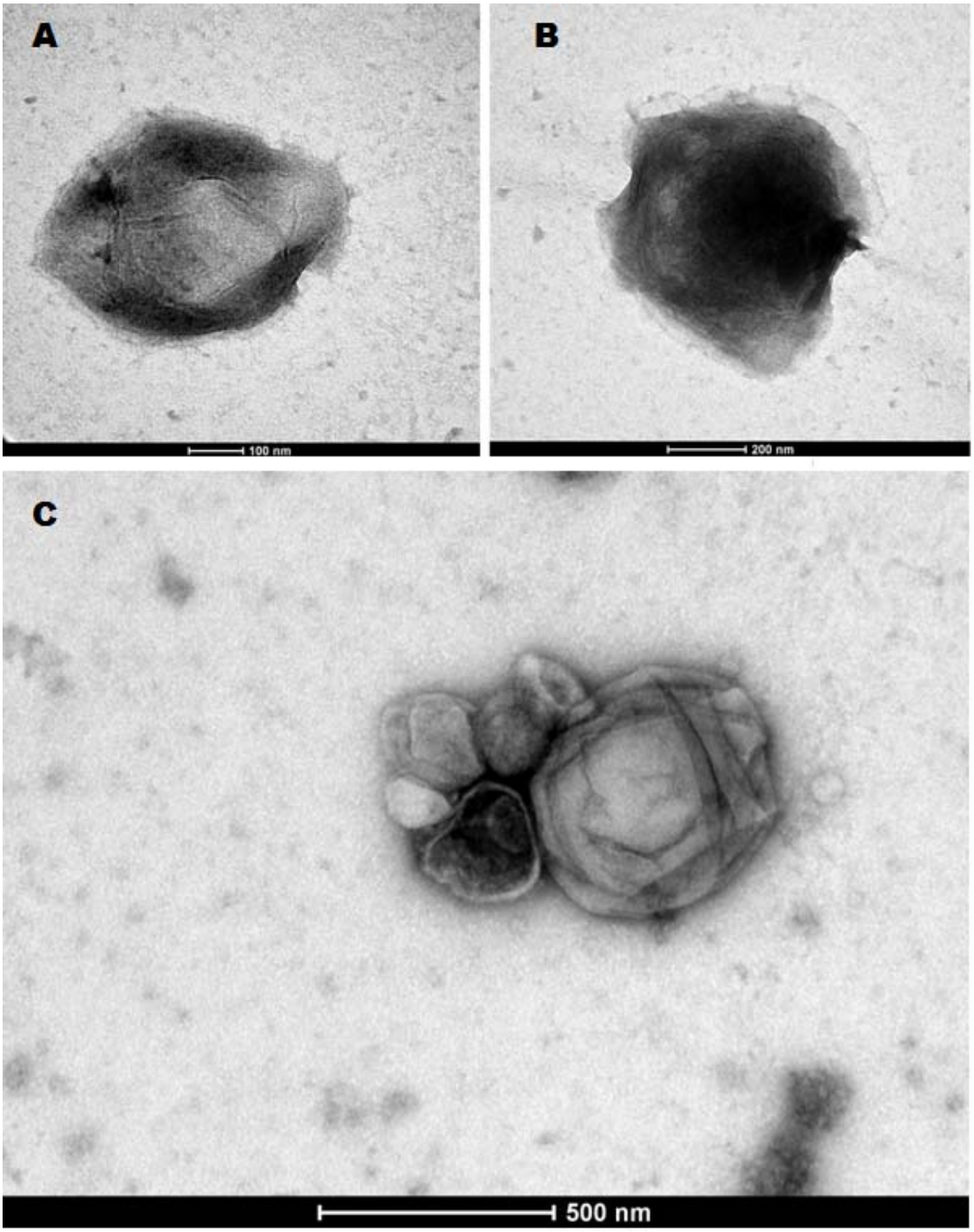

Figure 2. EM of giant viral particles isolated from CTVT after sucrose gradient purification. Images disclose a microbial system composed of ≤1 µm large particle and smaller satellite associated giant viral particles, Mimiviruses-sized. A-B) Details of the large microbial particle. C) The large particle and satellite giant viral particles.

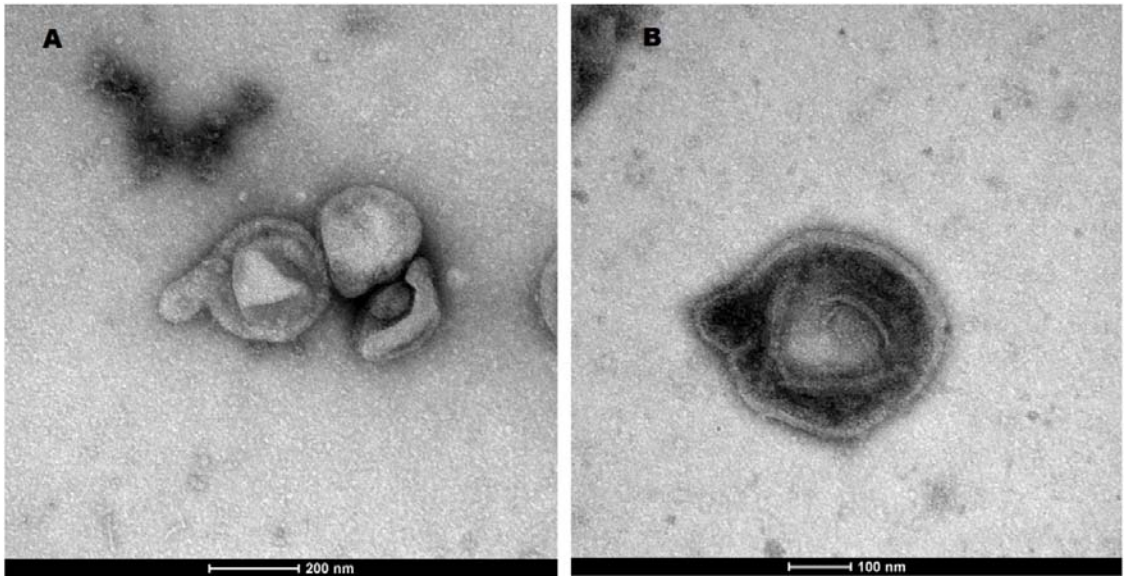

Figure 3. A-B) Detailed EM images of the satellite giant viral particles, isolated from CTVT.

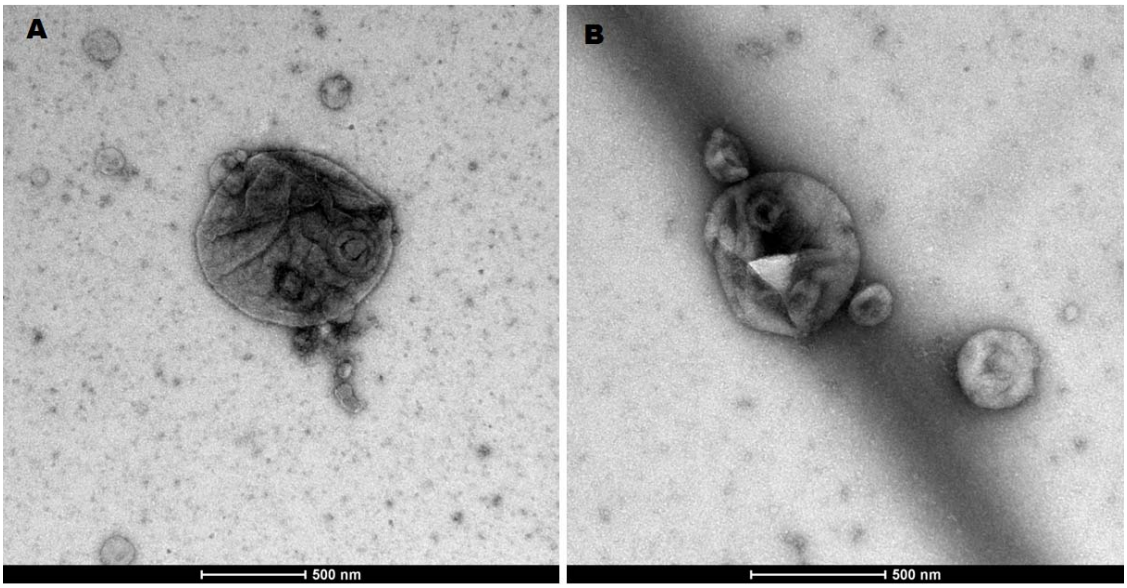

Figure 4. A-B) Satellite giant viral particles trafficking around and inside the large unicellular organism.

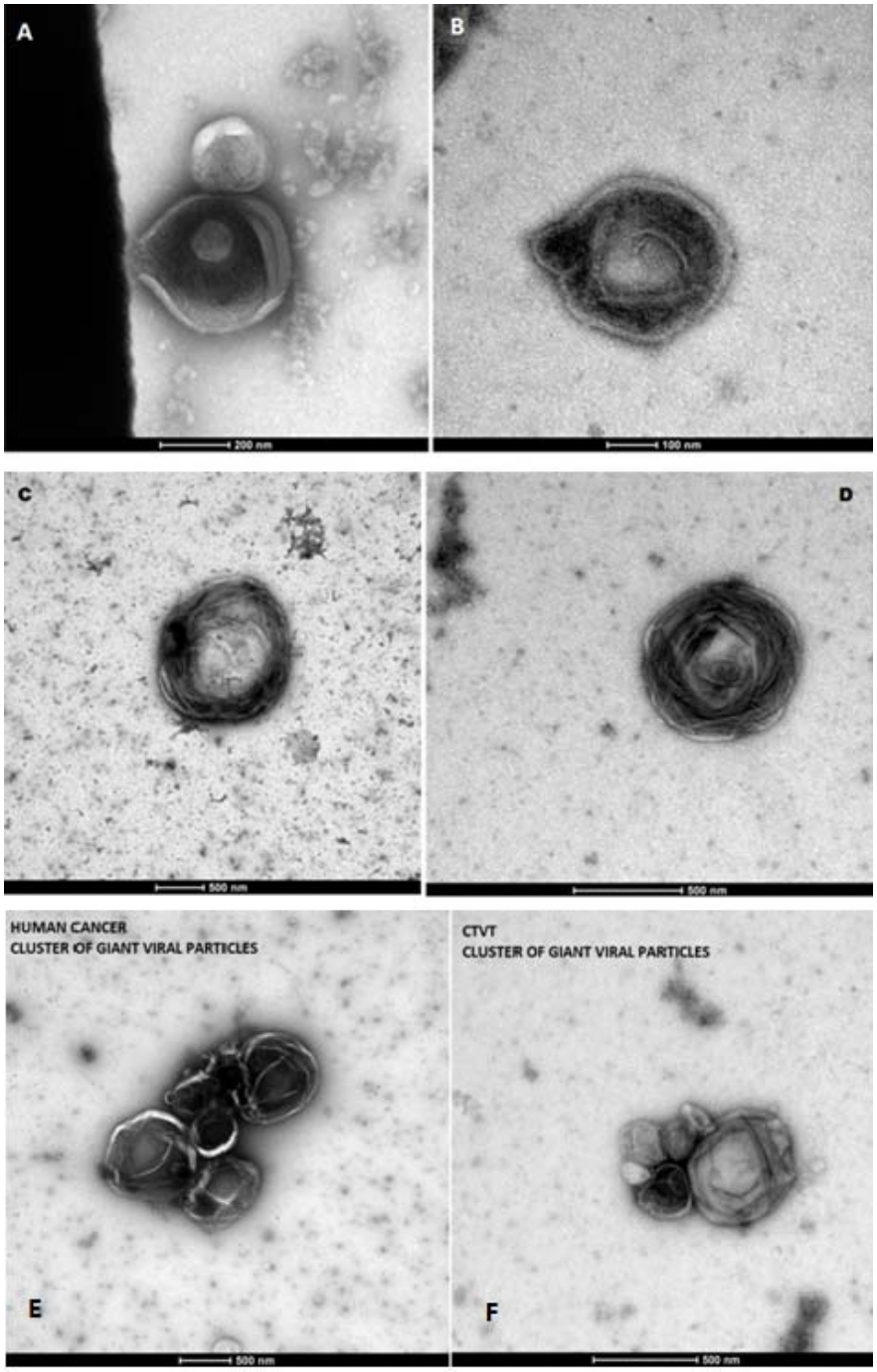

Figure 5. Comparison between human and CTVT giant viral particles. Human satellite giant viral particle isolated from T-cells leukaemia (A) have the same morphology as CTVT satellite giant viral particles (B). The large microbial unicellular organism isolated from human T-cell leukaemia (C) has the same morphology as CTVT large microbial cell-like entity (D). These oncogenic mammalian entities appear as a microbial system composed by a large unicellular organism surrounded by satellite giant viral particles, Mimiviruses-sized. E) Clusters of giant viruses isolated from human HPB- cells leukaemia F) CTVT cluster of giant viral particles.

Whole genome data processing and viral agent genome characterization

After sucrose gradient purification, total RNA was extracted from giant viral particles and c-DNA libraries were fully sequenced on Novaseq 6000 Illumina platform (100PE format). To remove the host genome contamination (dog genome) from the sample, the obtained reads (33214583) were firstly mapped on the *canis familiaris* reference genome and the remaining 2591909 reads, not matching with the dog genome (host), were used for *de-novo* assembly using Geneious (v2020.0.5) applying default parameters. Functional annotation against BLAST NCBI viral database and BLAST NR database was made with Blast2GO.

Similar to giant viruses infecting amoeba (28), the agent isolated from CTVT has a resulting mega-genome of ~1.17 MB, with a GC content of 46.13%. The genome of this agent exhibits exceptional features and puzzling origin, with a near-complete "ORF-an genome. In fact, more than 90% of predicted genes have never been described before, representing ORF-an https://mega.nz/#!yU5yTATT!j6jwGJI6Kj_G27D4Cgo9dR0Fl7qWfF8i1Ye9llMQ_jM

The number of uncharacterized genes remains high also extending the analysis against a nr database (Figure 5). The non ORF-an genes within the viral genome are retroviral core genes gag-pol. The retroviral core genes and reverse transcriptase depict this microbial agent as a retro-giant microbial entity, acutely transforming. Furthermore, only the fraction containing the giant viruses displays RT activity, while a filtered supernatant does not. This rules out the presence of filterable small dimension retroviruses and additionally dogs are the only animals in nature that lacks circulating archetypal retroviruses (29).

The genetic sequence of the giant virus isolated from CTVT is made available in the following link https://mega.nz/#!TRgyBAwZ!bb01EXhl-Q4XExi0Qcm869qW6GaNpN-1j8O23jmnME4

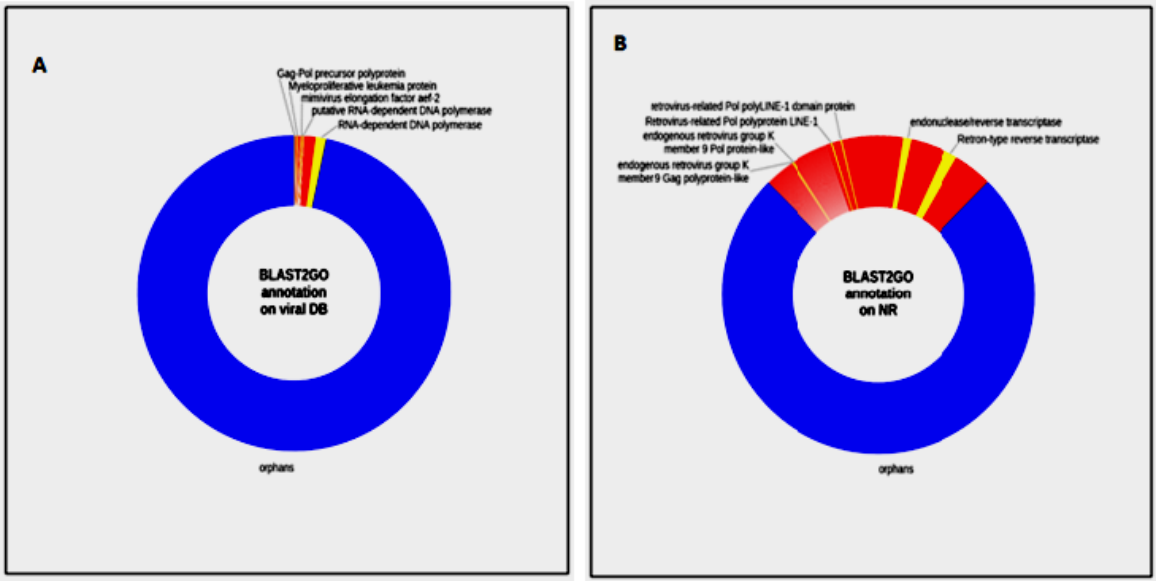

Figure 6. Genetic features of a giant virus isolated from CTVT. A) The 1.17MB viral mega-genome is a near-complete ORF-an genome, where the majority of genes are not present in any database. The blue color in the graph indicates the percentage of ORF-an genes. The non ORF-an genes, with homologues in database, are mainly retroviral core genes RT- Gag-Pol, orange-yellow shade. B) The number of uncharacterized genes remains high also when extending the analysis against a nr database, and the retroviral core genes (non ORF-an) are reconfirmed.

Tumorigenic Assay in vitro Focus Formation Assay-Transformation of NIH 3T3 cells

The giant viruses, isolated from CTVT, behave as acutely rapid transforming viruses, showing both transforming and immortalizing effects. In NIH 3T3 cells, the loss of contact inhibition and initial foci formation started after 48 hours post infection. In about a week, there were neoplastic foci in culture growing independently. The dynamics of malignant transformation in NIH 3T3 cells, induced by these large microbial agents, is reported in Table 1. It should be noted that only the fraction containing the giant viral particles induced neoplastic foci formation in NIH 3T3 cells, while a filtered supernatant did not. This ruled out the possible role of filterable viruses. Fulfilling Koch's postulates, the giant viral

particles could be re-isolated from infected NIH 3T3 cells that became cancerous, with the usual routine sucrose gradient.

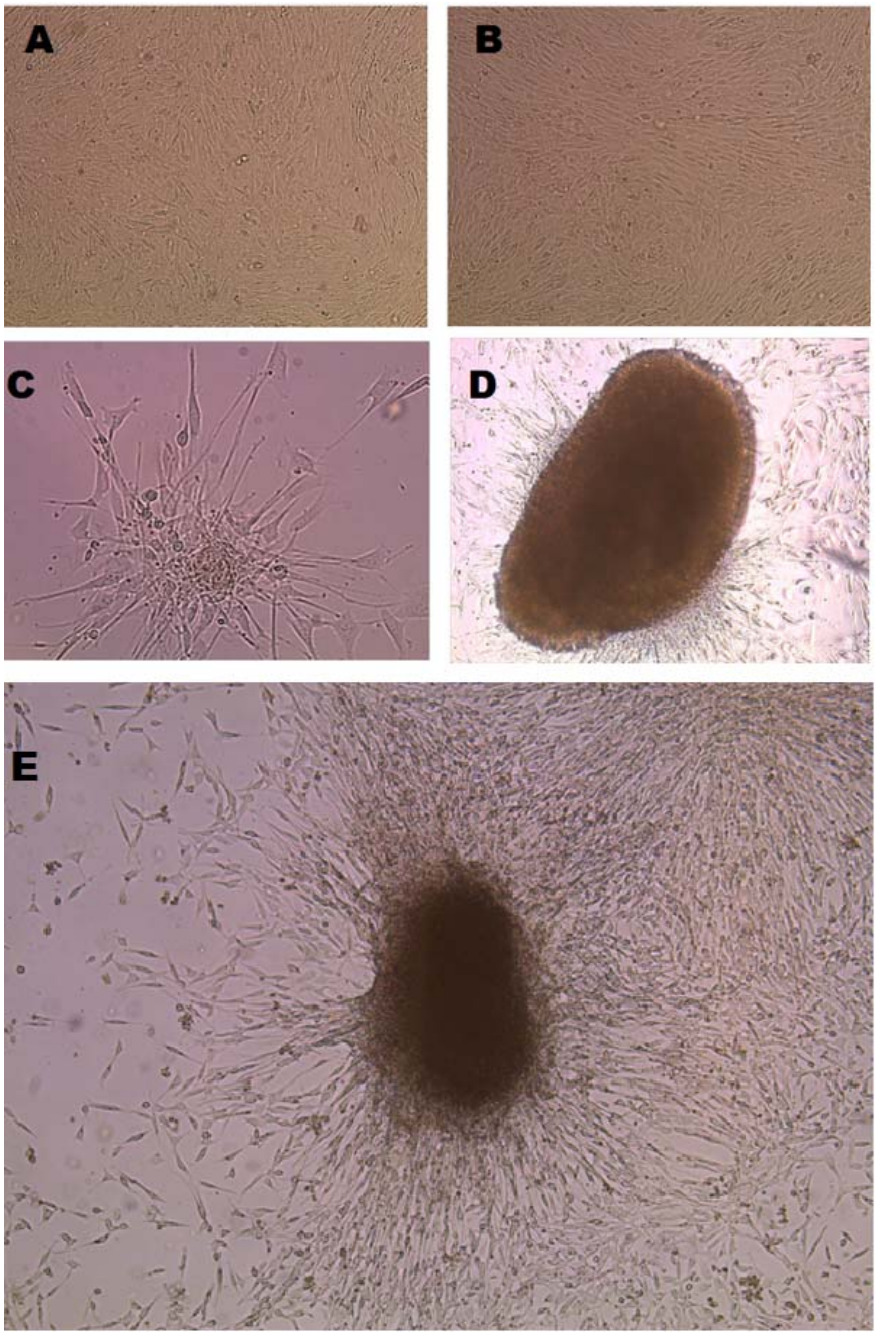

Table 1. A) Normal phenotype of non-infected NIH 3T3 cells. B) Normal phenotype of NIH 3T3 cells after infection with a filtered supernatant from CTVT. C) Initial neoplastic focus formation of NIH 3T3 cells infected by purified giant viral particles isolated from CTVT, 48 hours post infection. D- E) Typical neoplastic foci, ten days post infection.

Canine giant viruses and cancer formation in animals.

Experiments on animals were conducted in accordance to the EU Directives and Regulations relevant to animal welfare. The inoculation of a single dose of purified canine giant viral particles (150 µl) in a healthy dog, with no genital alterations, produced a penile cancer lesion, just after one week post-inoculum. After 4 weeks, tumour spread all over the penis and the cytology confirmed the typical histological pattern of Sticker's sarcoma (Fig. 7-8-9). This results suggest that CTVT is a contagious infectious cancer where the etiological agent, that initiates the typical CTVT cancerous lesions, is a transforming giant virus and not a cancer cell itself transmitted as an allograft, as previously believed.

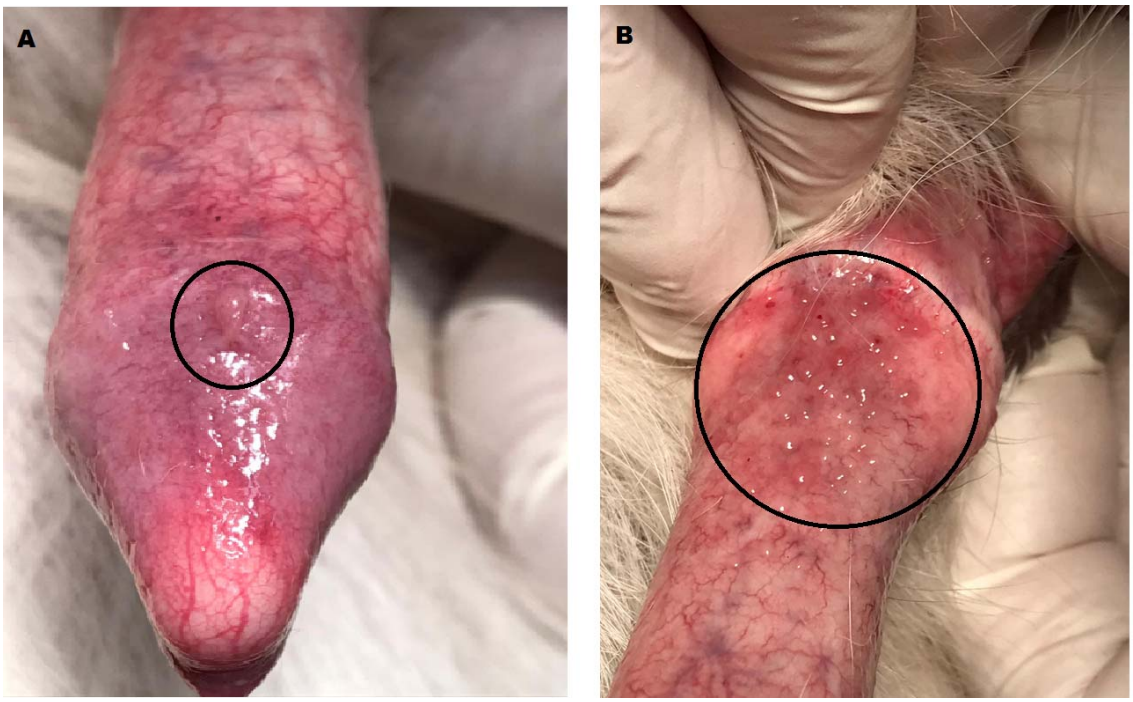

Figure 7. A) The circle indicates a cancerous lesion developed in the penis of a healthy dog one week after a single inoculum of giant viruses. B) At four weeks post-infection, cancerous vesicles and ulcers spread also in distal corpus of the penis. Note the vesicular lesions enclosed in the circle.

Figure 8. Penile CTVT induced by a single inoculum of giant viruses, 4-weeks post-infection. Vesicular cancerous lesions in the yellow square.

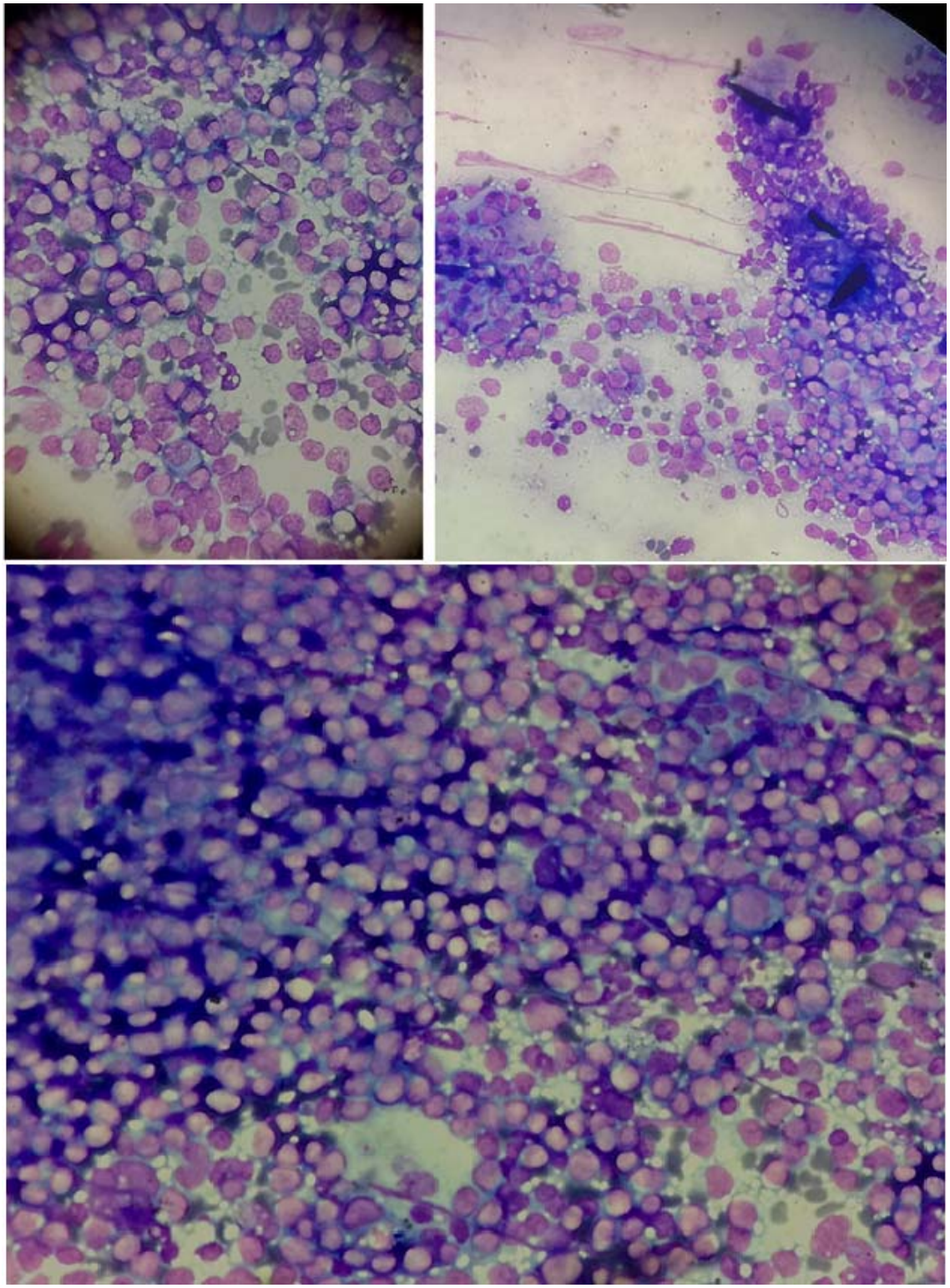

Figure 9. Cytology preparation, obtained by neoplastic lesions exfoliation, confirmed a new onset of CTVT in a healthy dog post giant viruses infection. The giant viruses initiated a penile cancer with the typical pattern characteristic of Sticker's sarcoma. Cytology smears were observed at 40X magnification, after May-Grünwald-Giemsa staining.

Discussion

We isolated from CTVT a microbial entity that appears to be halfway in between a classic oncogenic virus and an infectious cancer cell. This finding opens interesting scenarios on the origin and evolution of ancient cancer.

CTVT, Tasmanian Devil Facial tumour and Mya Arenaria soft shell clams leukaemia are the only examples of contagious cancer in nature (1-18). In particular, CTVT is the oldest contagious cancer and originated in dogs around 11.000 years ago. The current consensus view is that a transformed cell itself is transmitted and starts the tumour in a new animal, as a parasitic allograft**.** However, dogs or Tasmanian devils have no specific immune system impairment and they are able to reject transplants. Therefore, the mechanisms used by these cancer cells to escape the immune system in their hosts remain obscure to understand.

The evidences that only unfiltered tumour tissue could transmit cancer in healthy individuals, along with the failure to identify etiological viral agents in filtered cell-free extracts, raise doubts that the current epistemological definition of viruses and common filtration techniques caused the loss of giant microbial agents in transmissible cancer. In fact, classical virus isolation includes filtration through 0.2-µm-pore filters. However, these filters also often exclude large microbes. The recent discovery of Mimiviruses infecting amoeba highlighted how the use of a 0.45 µm filter prevents the recovery of giant viruses (19-24).

The model of contagious leukaemia in *Mya Arenaria* reinforces the hypothesis that we probably missed the presence of giant viruses in the aetiology of transmissible cancer. In fact, only the unfiltered hemolymph induces cancer in healthy clams, while a filtered supernatant does not (18).

Therefore, using a different strategy and avoiding filtration, we isolated from CTVT a microbial infectious unicellular organism and its satellites giant viral particles. Like giant viruses infecting amoeba, these mammalian agents have a gigantic size, a mega-genome and features of self-governing cell-like entities, but they manifest a different nature and their harvest from cancer specimens do not require amoeba co-cultures.

The transforming giant agent isolated from CTVT shows a distinctive genetic makeup and puzzling origin where more than 90% of predicted genes have never been described before, representing ORF-an. However, like analogous giant viruses isolated from human leukaemia that carry retroviral kinases and exhibit RT activity (25-27), these canine giant viral particles are characterized by the presence of reverse transcriptase and retroviral gag-pol genes. The human and canine giant viral particles point toward a new taxa of transforming microbial cell-like entities that display within their mega-genome the peculiar presence of retroviral core genes.

When analysing the retroviral nature of these oncogenic mammalian giant viral particles, the dog is a model particular interest since no substantiated reports of circulating retroviruses in dogs exist.

In fact, while near 8 percent of the human genome is derived from retroviruses, dogs in comparison display a significantly lower ERV presence, with only 0.15% of the genome recognizably of retroviral origin, with study suggesting that most canine ERVs lineages ceased replicating long ago (29-32).

Strangely, dogs have missed a history of retroviruses infecting their germ line and are the only mammals lacking circulating infectious retroviruses. Humans have HIV and HTLV; mice have circulating retroviruses; cats have FELV and FIV; birds have ALV, but dogs are an exception and are the only animal that lacks circulating exogenous retroviruses. Instead,

the canine giants viral particles, ten times bigger than a conventional retrovirus, can be isolated, purified, examined at EM, tested for their RT activity and stored as purified microbial aliquots for downstream applications. The mysterious absence of circulating retroviruses in dogs as a precondition and the failure of a filtered CVTV supernatant to transform, ruled out the presence of filterable small size viruses. The retroviral genes assembled perfectly within the giant viruses mega-genome and only the fraction containing the giant viral particles had RT activity and transformed.

The evidence in *Mya Arenaria* contagious leukemia that only the unfiltered lysate had the highest RT activity fits perfectly the picture and strongly suggests the presence of oncogenic giant viruses with a retroviral nature also in this form of contagious tumor.

Challenging the current believes that a somatic cancer cell, transmitted as a parasitic allograft, starts contagious tumours in the wild, the giant viruses inoculated in a healthy dog initiated the typical Sticker's sarcoma in one week.

Conclusion

The discovery of giant microbial entities in contagious cancer in the wild proposes a paradigm shift from a somatic cancerous cell allograft to a self-governing microbial cell-like entity, a simplified version of its eukaryotic counterparts, specialized in carcinogenesis.

In order to save the Tasmanian devils, that share with the dogs the same cancer tale, a different approach would be wise.

The possibility of treating some forms of cancer like an infectious disease by targeting a microbe and its hundreds proteins could become a reality. Stray dogs and Tasmanian devils are opening the door to a preventive cancer vaccine in the wild and humans.

Material and Methods

Isolation of giant viral particles from CTVT on sucrose gradient

CTVT tumour biopsies was washed with 1x PBS, homogenized at 4C, lysed (vortexed) with 2.5 ml PBS in the presence of 25µl of protease inhibitor cocktail (Abmgood, Richmond BC, Canada). Cell suspension was vortexed and incubated a 4°C for 30 minutes. The resulting crude extract was centrifuged at 3,000 rpm for 5 minutes. The pellet was discharged. The resulting supernatant was collected and slowly dripped over 9 ml of a 35-30-25% sucrose gradient (Sigma, Milan, Italy) and centrifuged at 10,000 rpm for 2 h in a 15 ml Polyclear Centrifuge tubes (Seton USA). Once a visible white disk, corresponding to 25% sucrose fraction, was observed, the fraction was collected after centrifugation at 14,000 rpm for 30 min, at 4°C.

Electron microscopy of the giant viral particles

25 µl of purified viral aliquots were placed on Holey Carbon film on Nickel 400 mesh.After staining with 1% uranyl acetate for 2 minutes the sample was observed with a Tecnai G (FEI) transmission electron microscope operating at 100 kV. Images were captured with a Veleta (Olympus Soft Imaging System) digital camera.

Whole genome Sequence, data processing and viral agent genome characterization

After sucrose gradient purification, total RNA was extracted from giant viral particles (Lucigen RNA extraction kit). Total RNA underwent a further purification step through Zymo Research columns and c-DNA libraries were fully sequenced at BMR Genomics (Padova, Italy, www.bmr-genomics.it) on Novaseq 6000 Illumina platform (100PE format). To remove the host genome contamination (dog genome) from the sample, the obtained reads (33214583) were firstly mapped by using hisat2 aligner (v2.1.0) (I) on the *canis familiaris* reference genome (Canis_familiaris.CanFam3.1.dna.toplevel.fa - Ensembl release 98). Subsequently, paired unmapped reads where selected applying samtools v1.7 (II) (samtools view command exploiting the flags 77 and 141) and reconverted into FASTQ format. The remaining 2591909 reads, not matching with the dog genome (host), were used for *de-novo* assembly using Geneious (v2020.0.5) applying default parameters. Statistics on the assembled contigs were calculated by using quast (v4.6.3) (III), considering only contigs longer than 500 bp. Functional annotation against BLAST NCBI viral database (ftp://ftp.ncbi.nih.gov/blast/db/ref_viruses_rep_genomes.tar.gz – version December 2019) and against BLAST NR database was made with BLAST 2GO.

Focus Formation-NIH 3T3 cells Transformation Assays, post viral infection

NIH 3T3 cells (ATCC -lot 700009353) were propagated in DMEM plus 10% FBS. 3×10^5 NIH 3T3 cells per 10 cm culture dish were seeded with 10 ml of NIH 3T3 medium and incubated O/N at 37 °C, 5% CO_2 for infection the next day. For the seeding cells were kept at 50-60% confluence. The following day, the medium, from NIH 3T3 cells to be infected, was aspirated and replaced with 9 ml of fresh regular NIH 3T3 medium, 1 ml of purified viruses aliquots and polybrene at a final concentration of 6 µg/ml. The infected cells were incubated O/N at 37°C, 5% CO_2. Only one round of infection was performed and the virus-containing medium was replaced with regular NIH-3T3 medium. Uninfected NIH-3T3 cells and NIH

3T3 cells infected with a filtered supernatant were used as controls. Phenotypic changes were valuated every day, for 2-3 weeks and medium was replaced as necessary.

Tumorigenicity experiments in dogs

For the *in vivo* tumorigenicity assay, all protocols were performed according to the Standard Operating Procedures and Animal Care Legislative decree N. 26/2014. A 10 year-old male mongrel dog, 25 kg weight, was selected for our experimentation. To rule out systemic pathology at the time of the enrolment, the animal underwent a preliminary screening that included blood count test, leishmania and ehrlichia serological tests and a clinical examination of the genital tract. In order to reproduce the natural history of CTVT and simulate the micro-lesions occuring during coitus, a light scraping with a cover slip was carried out on the dorsal face of the glans penis (near the lesion that appeared in fig 7 A). Subsequently, a single dose of purified giant canine viral particles (150 µl) was inoculated into the prepuce and the preputial orifice was keep closed for a few minutes, before releasing the animal. The animal follow-up was carried out on a weekly basis, monitoring the general clinical conditions and tumour formation. The diagnosis of CTVT was made by inspection and by cytology exfoliation of vesicular lesions, that appeared already in the first week of inoculation. Cytology smears were observed at 40X magnification, after May-Grünwald-Giemsa staining.

Data availability

The entire electron microscopy collection, specimens and stored purified viral aliquots are available to be examined; please contact the corresponding author.

Competing interests

No competing interests were disclosed.

Grant information

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