THE CANCER WHICH SURVIVED: Insights from the genome of an 11,000 year-old cancer

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ABSTRACT
The canine transmissible venereal tumour (CTVT) is a transmissible cancer that is spread between dogs by the allogeneic transfer of living cancer cells during coitus. CTVT affects dogs around the world and is the oldest and most divergent cancer lineage known in nature. CTVT first emerged as a cancer about 11,000 years ago from the somatic cells of an individual dog, and has subsequently acquired adaptations for cell transmission between hosts and for survival as an allogeneic graft. Furthermore, it has achieved a genome configuration which is compatible with long-term survival. Here, we discuss and speculate on the evolutionary processes and adaptions which underlie the success of this remarkable lineage.

INTRODUCTION
The canine transmissible venereal tumour (CTVT) (Figure 1A) is a cancer that first emerged as a tumour affecting an individual dog that lived about 11,000 years ago [1-3]. Rather than dying together with its original host, the cells of this cancer are still alive today, having been passaged between dogs by the transfer of living cancer cells during coitus (Figure 1B). The genome of CTVT, which has recently been sequenced, bears the imprint of the evolutionary history of this extraordinary cell lineage [1]. Furthermore, the genome variation captured in global CTVT populations has highlighted some of the unique adaptations that have driven this lineage to become the longest-living and most prolific cancer known in nature. This "cancer which survived" is a remarkable biological entity which illustrates that evolution can drive a transition from mammalian somatic cell to obligate colonial parasite.

The canine transmissible venereal tumour: origins of a global parasite
CTVT is a sexually transmitted cancer that affects dogs and usually manifests clinically with tumours associated with the external genitalia of both male and female animals (Figure 1A). Although CTVT first appeared in the veterinary literature at least two hundred years ago [4], its uniqueness as a transmissible cancer was not noted until much later [2,3,5-7]. CTVT is endemic in at least ninety countries worldwide across all inhabited continents and its distribution is linked to the presence of free-roaming dogs [8].

Although CTVT is found worldwide, the patterns of genetic identity detected in tumours located on different continents indicate a single clonal origin for the disease [2,3]. Analysis of a mutational process with clock-like features, as well as comparison of microsatellite variation between tumours and between tumours, dogs and wolves, suggest that the lineage first arose as a cancer several thousand years ago [1-3]. By searching for genetic variation present in the CTVT genome and comparing it with genotypes associated with specific traits in modern canids, a picture of the "founder dog" that first spawned CTVT has emerged [1-3]; it appears that this individual was more closely related to modern dogs than modern wolves and had relatively low levels of genomic heterozygosity. This animal was probably of medium or large size with an agouti or solid black coat. The XO karyotype and genotype found in CTVT tumours precludes conclusions about the founder animal's gender [1,9].

CTVT probably first arose from a somatic cell, possibly a tissue macrophage or a dendritic cell [10,11], of this "founder animal" via evolutionary processes that are common between all cancers. The life-history of a cancer is generally characterised by successive waves of clonal outgrowth, driven by the acquisition of positively selected "driver" mutations [12]. The molecular processes promoted by driver mutations can shed light on the biological pathways underlying cancer, such as proliferative autonomy, resistance to cell death and genomic instability [13]. CTVT shares a number of putative driver mutations with human cancers, some of which possibly occurred in the original CTVT tumour. These include a rearrangement involving *MYC*, homozygous deletion of the *CDKN2A* locus, homozygous loss of *SETD2* and a rearrangement involving *ERG* that creates a potential in-frame *NEK1-ERG* fusion gene [1,5]. There is, however, no evidence to suggest that the original CTVT or its host were particularly extraordinary; we cannot know if the original CTVT was metastatic in its founder dog, or even if the original CTVT was the cause of its founder's death. Nevertheless, we presume that a series of highly improbable events next triggered CTVT to become a transmissible cancer (Table 1).

Crossing the gaps
Cancers frequently acquire features that cause cells to depart from a primary tumour and establish new tumours in distant sites of the body via a process of metastasis. CTVT, however, has acquired adaptations for the transmission of cancer cells to new hosts. The family Canidae may have been particularly at risk for the establishment of a sexually-transmitted cancer due to the existence of the long-lasting coital tie that is peculiar to this group. The coital tie may last for up to thirty minutes, and may lead to injuries to the genital mucosa; such conditions may thus provide an exceptional opportunity for the exchange of cancer cells between individuals [14]. Despite the potential for mating between dogs and wild canids, including wolves and coyotes, CTVT has not been reported within wild canid populations [8]. CTVT tumours are also occasionally found affecting non-genital regions, most commonly skin, nasal cavity, lymph node, eye and mouth [8]. As these sometimes occur without genital involvement [15-17], this suggests that there may be non-coital routes of CTVT transmission, possibly involving licking, sniffing or parturition.

Transmissibility has presumably had consequences for CTVT genome evolution. Direct transmission of cancer cells may select for loss of cell adhesion; indeed, CTVT tumours are typically highly friable [18-20]. Furthermore, CTVT tumours are delicately encapsulated and bleed readily upon contact [20], presumably optimised for the release of CTVT cells during the friction involved in coitus. A genetic imprint of the CTVT transmission cycle was identified with the discovery that approximately forty percent of mutations in CTVT were caused by exposure to ultraviolet (UV) light from the sun [1]. Although UV mutagenesis would be expected to impact only the surface layer of cells of an ulcerated externally-facing tumour, it is these very cells, indelibly marked with a UV imprint, which have sustained the lineage by transfer to new hosts.

The requirement for existence within an external compartment with ready access to new hosts may be a barrier for the emergence of naturally transmissible cancers. However, a variety of routes of cancer cell transmission could be envisaged, and depend on the behaviour and biology of the host species. The Tasmanian devil facial tumour disease, the only other known naturally occurring transmissible cancer, is transmitted by biting [21,22], exploiting the facial biting behaviour that this species engages in during aggressive interactions. Furthermore, a transmissible cancer in a laboratory population of hamsters was transmissible by cannibalism and mosquitos [23-25]. Cancer cells have also rarely been reported to have spread between two humans within a variety of contexts, including surgical accident, organ transplant, in utero and during experimental treatments [26-31].

Once deposited within the breached mucosa of another animal, CTVT must next overcome perhaps the most potent obstacle facing transmissible cancers: the immune system.

Evading the barriers

Although all cancers, including those that remain within a single host, may have acquired adaptations to escape immune destruction, transmissible cancers are able to escape the immune system as an allogeneic graft. The highly potent immune response to allogeneic grafts is primarily mediated by direct allorecognition of foreign major histocompatibility complex (MHC) molecules by the graft recipient's T cells [32]. Although MHC molecules are normally expressed by all nucleated cells, both CTVT and DFTD cells have lost expression of MHC molecules, presumably via a process of immunoselection [2,33-36]. Similarly, many human cancers modulate MHC molecule expression as a mechanism to escape immune detection [37].

The mammalian immune system has specific mechanisms to detect cells which are not expressing MHC molecules. Natural killer (NK) cells are specialised lymphocytes which become cytotoxic when activated by "missing self", i.e. absent MHC. The mechanisms whereby transmissible cancers escape NK cell killing remain unclear [38]. The recruitment of an immunosuppressive tumour microenvironment leading to immune tolerance or anergy may be an important feature in transmissible cancer immune escape [33,39-41]. This suggestion is supported by the observation that CTVT rarely metastasises, thus departing from the established tumour microenvironment, except in immunosuppressed hosts and newborn puppies [42,43].

Early observations of CTVT revealed that experimentally transplanted tumours frequently undergo immune-mediated spontaneous regression two to six months after transplantation [44-46]. This, however, contrasts with naturally occurring CTVT, where spontaneous regression has not been consistently reported [14,47,48]. The immune response to CTVT may be influenced by the site of tumour transplantation (experimentally transplanted CTVT tumours are usually injected subcutaneously) and the concurrent presence of injuries and inflammation. It is also possible that there is variation in susceptibility to CTVT within the dog population that influences clinical progression and disease course [49]. This is supported by the observation that CTVT is usually found at low prevalence within affected dog populations [8].

Thousands of years of passaging between allogeneic hosts has presumably exerted powerful immunoselective pressures on CTVT. Signatures of this process may be present in the CTVT genome, possibly acting to prevent mutation of cell surface antigens. However, CTVT has possibly faced yet another selective challenge: maintaining its genome and cellular integrity over thousands of years despite the irreversible accumulation of mutations.

Surviving the millennia

Genetic variation, caused by the accumulation of somatic mutations, is the raw material upon which natural selection operates to drive the outgrowth of cancer [12]. Thus, genome instability and loss of DNA repair pathways have been described as "enabling characteristics" of cancer [13], and most human cancers carry a few thousand point mutations as well as structural variants and aneuploidy [12,50]. Most of these mutations are considered to be selectively neutral, captured in the cancerous clone by hitchhiking together with a small number of positively selected driver mutations. Interestingly, negative selection, operating to curb the accumulation of mutations that decrease fitness, has not been robustly detected in cancer [51-53].

The exceptionally long lifespan of CTVT as a cancer raises the possibility that the accumulation of mutations has become a burden rather than an advantage in this lineage. Indeed, the CTVT genome has acquired approximately 1.9 million somatic substitution mutations, as well as thousands of

structural rearrangements, copy number changes and retrotransposon insertions [1]. Interestingly, however, despite the enormous number of mutations and marked aneuploidy, the genomic rearrangements and microsatellite alleles observed in CTVT tumours collected from different continents are remarkably similar [1-3,7,9,54]. It is possible that CTVT has maintained or activated DNA repair and telomere stabilisation mechanisms that safeguard its genome against further mutation and instability. Additionally, given the large mutation burden already carried by CTVT, its genome may be particularly sensitive to further mutation such that negative selection acts to maintain stability. It is interesting that the oldest human cancer lineage, the HeLa cell line, which has continued to survive by passaging in laboratory cell culture for more than sixty years, also appears to have a relatively stable genome in terms of point mutation [55].

The occasional capture of mitochondrial DNA from its hosts appears to be one mechanism acquired by CTVT to support long-term survival [56] (Figure 2). The mitochondrial genome is gene rich, has a particularly high mutation rate [52], and encodes proteins involved in energy metabolism. The observation that mitochondrial DNA in CTVT is not clonal, but rather appears to have been acquired by periodic horizontal transfer from dogs, suggests that replacement of CTVT mitochondrial DNA, which presumably was carrying large numbers of possibly deleterious mutations, provided a selective advantage to the lineage (although we cannot exclude the possibility that CTVT acquired host mitochondrial DNA via purely neutral processes) [56]. Shuttling of mitochondria between cells may be more common than previously appreciated, as mitochondrial DNA has been observed to exchange between human cells *in vitro* as well as between normal and cancer mouse cells *in vivo* [57,58]. Although horizontal DNA transfer is in general a rare phenomenon in the animal kingdom, it has been occasionally described [59]. In one interesting example, horizontal DNA transfer was observed in ancient asexual bdelloid rotifers, and it was suggested that capture of environmental DNA may compensate for absence of sexual recombination and loss of gene function due to mutation [60,61].

Influencing each other

Parasites sometimes directly influence their hosts' behaviour or physiology so as to optimise transmission to new hosts [62]. Given that CTVT has co-existed with its host for millennia, one could speculate that it may have acquired mechanisms to manipulate its hosts' sexual receptiveness, oestrus cycle timing or smell preferences to enhance its chances of transmission. Interestingly, it appears that oestrogen receptor expression differs between the vaginal epithelium of CTVT-affected females and control females during certain stages of the oestrus cycle, suggesting that CTVT may modulate the local tissue environment [63]. Furthermore, there is evidence that CTVT may stimulate erythropoietin production by its host, or possibly directly produce erythropoietin via a paraneoplastic process [14]; a consequent production of red blood cells may compensate for blood loss via tumour bleeding, thus protecting its host from anaemia. Given that they carry a conspecific genome, transmissible cancers may be uniquely placed to directly manipulate the biology of their hosts.

CONCLUSION

Transmissible cancers are very rare in nature; indeed, naturally occurring transmissible cancers have been described only twice. Given that cancer itself is a common condition, both in humans and animals, it is clear that there is a very low probability for a cancer to develop into a transmissible form. In order to become transmissible, a cancer must acquire adaptations both to support the physical transmission of living cancer cells between hosts, and to escape the immune system within an allogeneic host. Once transmissible, a cancer must acquire a genome configuration that is compatible with long-term survival. The genome and biology of CTVT have started to illuminate how this particular cancer has become transmissible, and future research may reveal fundamental features that drive cancers to become long-lived parasites.

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Describes the sequencing and analysis of two CTVT genomes. The authors found that CTVT has acquired approximately 1.9 million somatic substitution mutations and bears evidence of exposure to ultraviolet light. Despite the presence of extensive aneuploidy, the two CTVT tumours were found to be remarkably genetically similar. CTVT was shown to have first originated from a dog with low genomic heterozygosity that may have lived about 11,000 years ago.

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An important study confirming that the agent causing CTVT is the tumour cell itself. The authors analysed CTVT tumours and matched hosts across a panel of genetic markers including major histocompatibility (MHC) genes, microsatellites and mitochondrial DNA. They showed that, in each case, the tumour is genetically different from its host, but that all the tumours share a single clonal origin around 200 to 2500 years ago.

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This study uses microsatellite length differences and microarray-based comparative genomic hybridization (aCGH) to determine evolutionary relationships between CTVT samples from different continents. The analysis revealed remarkable similarity between all CTVT tumours. Additionally, the study indicated that the tumour arose from either dogs or wolves more than 6000 years ago. The common ancestor of today's CTVT tumours was, however, found to live within the last few hundred years.

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Table 1. The cancer which survived.
Transmissible cancers must acquire adaptations that allow them to survive as long-lived cell lineages. Summary of barriers to the emergence of transmissible cancers, and speculation on possible mechanisms acquired by CTVT to overcome these.

Adaptations of transmissible cancers	Speculation on possible mechanisms used by CTVT
Transmission between hosts	Tumour friability and ulceration, growth in external compartment, transmission during extended and often injurious canine coitus
Immune evasion	Down-regulation of MHC molecules from the cell surface, NK cell avoidance, recruitment of immunosuppressive microenvironment, inflammation
Maintenance of genome integrity	Maintenance or activation of DNA repair processes, telomere stabilisation, negative selection, mitochondrial genome capture from hosts

Figure 1. Canine transmissible venereal tumour (CTVT).
(A) CTVT causes tumours most often associated with the external genitalia of both male (left) and female (right) dogs.
(B) CTVT first emerged from the somatic cells of the "founder dog" about 11,000 years ago. Since then, it has been transmitted between individual dogs by the allogeneic transfer of living cancer cells.

Figure 2. Horizontal transfer of mitochondria.
The finding that CTVT mitochondrial genomes derived from tumours in different dogs do not share a clonal origin led to the proposal that CTVT cells periodically capture mitochondria from their hosts [56]. CTVT cells (grey) are shown acquiring mitochondrial genomes from host cells (red and blue). Over time, the original CTVT mitochondrial genome (black), which presumably carried a large mutation burden, is replaced by the acquired host mitochondrial genomes (red and blue) via a process of genetic drift or positive selection.
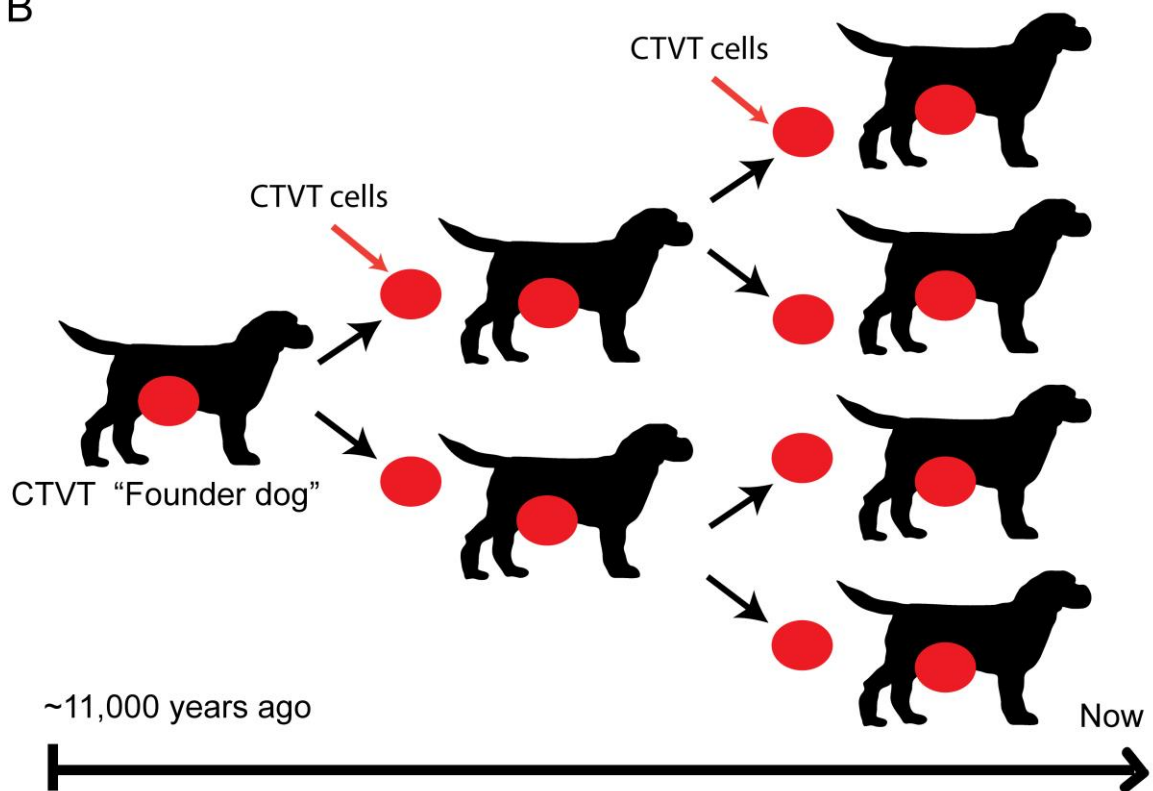
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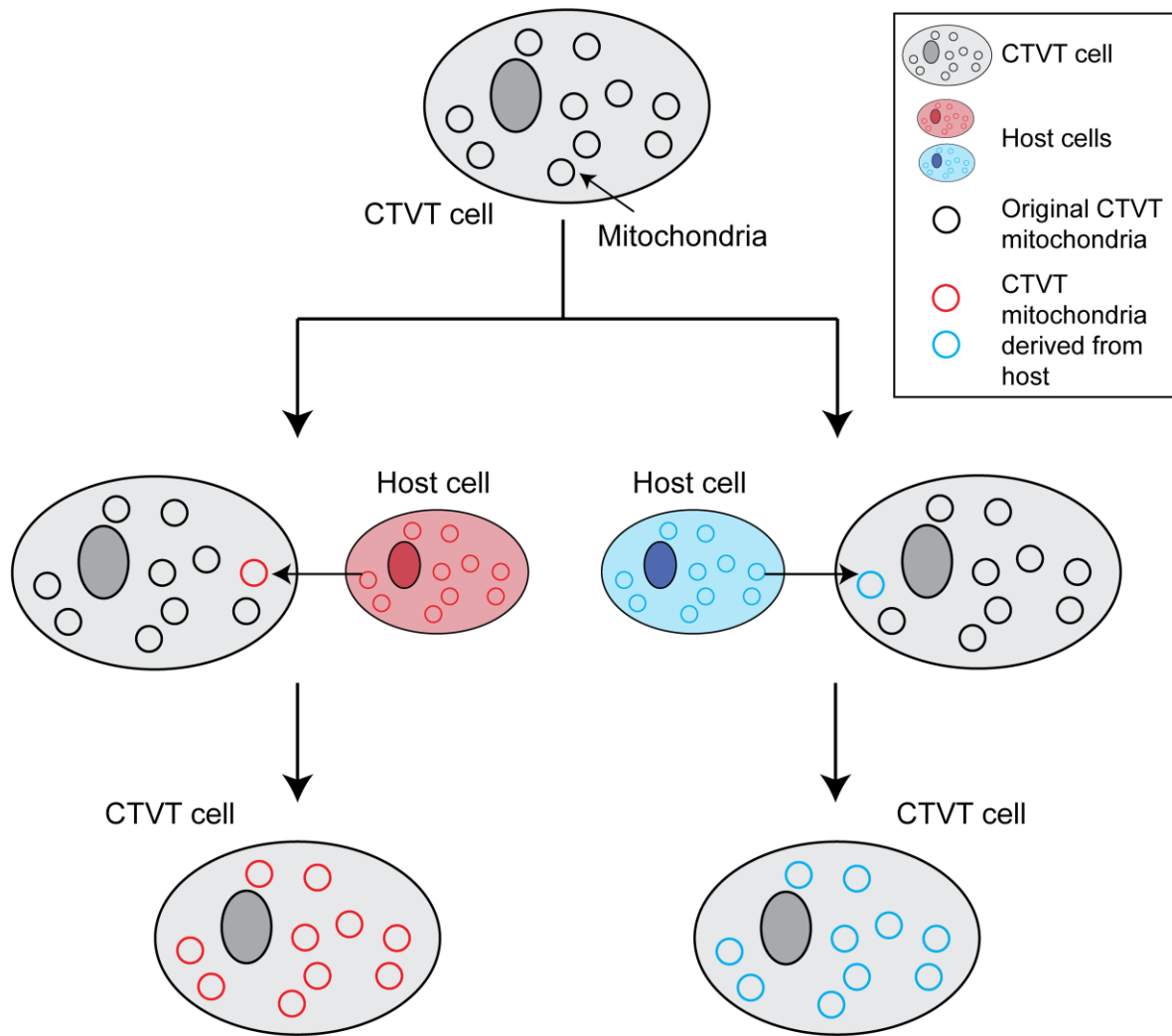
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