A second transmissible cancer in Tasmanian devils

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ABSTRACT

Clonally transmissible cancers are somatic cell lineages that are spread between individuals via the transfer of living cancer cells. There are only three known naturally occurring transmissible cancers, and these affect dogs, soft-shell clams and Tasmanian devils respectively. The Tasmanian devil transmissible facial cancer was first observed in 1996, and is threatening its host species with extinction. Until now, this disease has been consistently associated with a single aneuploid cancer cell lineage that we refer to as DFT1. Here we describe a second transmissible cancer, DFT2, in five devils located in southern Tasmania in 2014 and 2015. DFT2 causes facial tumours that are grossly indistinguishable but histologically distinct from those caused by DFT1. DFT2 bears no detectable cytogenetic similarity to DFT1 and carries a Y chromosome, which contrasts with the female origin of DFT1. DFT2 shows different alleles to both its hosts and DFT1 at microsatellite, structural variant, and major histocompatibility complex (MHC) loci, confirming that it is a second cancer that can be transmitted between devils as an allogeneic, MHC-discordant graft. These findings indicate that Tasmanian devils have spawned at least two distinct transmissible cancer lineages and suggest that transmissible cancers may arise more frequently in nature than previously considered. The discovery of DFT2 presents important challenges for the conservation of Tasmanian devils and raises the possibility that this species is particularly prone to the emergence of transmissible cancers. More generally, our findings highlight the potential for cancer cells to depart from their hosts and become dangerous transmissible pathogens.

SIGNIFICANCE STATEMENT

Transmissible cancers are somatic cell lineages that are spread between individuals via the transfer of living cancer cells. Only three transmissible cancers have been reported in nature, suggesting that such diseases emerge rarely. One of the known transmissible cancers affects Tasmanian devils, and is threatening this species with extinction. Here we report the discovery of a second transmissible cancer in Tasmanian devils. This cancer causes facial tumours that are grossly indistinguishable from those caused by the first-described transmissible cancer in this species; however, tumours derived from this second clone are genetically distinct. These findings indicate that Tasmanian devils have spawned at least two different transmissible cancers, and suggest that transmissible cancers may arise more frequently in nature than previously considered.
INTRODUCTION

Clonally transmissible cancers are somatic cell lineages that are contagious between individuals via the transfer of living cancer cells. Only three transmissible cancers have been observed in nature, and these cause Tasmanian devil facial tumour disease (DFTD), canine transmissible venereal tumour (CTVT) and soft-shell clam disseminated neoplasia respectively (1, 2). Each of these clones originated in a “founder animal” whose somatic cells acquired changes that drove carcinogenesis as well as adaptations for transmission and long-term survival (3). The rarity of transmissible cancer lineages in nature, despite the ubiquity of cancers that remain in one individual, suggests that the emergence of such clones is extraordinarily improbable.

Tasmanian devils (Sarcophilus harrisii) are iconic marsupial carnivores endemic to the Australian island state of Tasmania. DFTD is spread between Tasmanian devils by biting and causes tumours usually on the face or inside the mouth (Fig. 1) (4, 5). DFTD readily metastasises, and the disease usually causes death of affected animals within months of the appearance of symptoms (4, 5). Since it was first observed in 1996 in north-east Tasmania, DFTD has spread through most of Tasmania and has triggered widespread devil population declines (Fig. 1A) (4, 6, 7). The species was listed as endangered by the International Union for Conservation of Nature in 2008 (8).

DFTD has been associated with a single cancer clone that is genetically distinct from its hosts (9). This DFTD lineage carries a distinctive aneuploid karyotype notable for the presence of four rearranged marker chromosomes (9). DFTD tumours share identical alleles at major histocompatibility complex (MHC) loci (10), and the survival of this lineage as an allogeneic graft in MHC-discordant hosts is at least partly mediated by down-regulation of cell surface MHC molecules (11). DFTD tumours collected from different geographical locations in Tasmania share identical alleles at microsatellite loci (10, 12) and the whole genome sequences of two distantly located DFTD tumours were found to share the majority of their genetic variation (13). The DFTD clone has been closely monitored during its epidemic sweep through Tasmania (Fig. 1A) (13-16).

Here we report the discovery of a second transmissible cancer in Tasmanian devils. This second cancer, DFT2, manifests as facial tumours that are grossly indistinguishable from those caused by the original DFTD clone, now designated DFT1. However, DFT2 bears no detectable genetic or cytogenetic similarity to DFT1 and the tumours that it causes are histologically distinct. This finding indicates that DFTD tumours can be caused by at least two distinct transmissible cancer clones. Given the rarity of known transmissible cancer clones, it is remarkable that a second clone has emerged in Tasmanian devils. These findings suggest that Tasmanian devils may be particularly prone to this type of disease, or alternatively, that transmissible cancers are generally more common than previously detected. The discovery of DFT2 changes our perception of transmissible cancers as exceptionally rare and bizarre natural occurrences, and challenges our understanding of the processes that cause somatic cells to depart from their hosts and become transmissible cancer lineages.

RESULTS

Gross and histological characteristics of DFT2

DFTD was first observed in north-east Tasmania in 1996, and rapidly spread south and west across the island (Fig. 1A). Tasmanian devils with signs of DFTD were first recorded in the Channel area, a ~550 km² peninsula in Tasmania’s south-east, in December 2012. Since then, there have been twelve confirmed DFTD reports in the area (Fig. 1A, Table S1). Tumours in these animals ranged in appearance from small foci involving the oral mucosa and/or facial skin (e.g. JV, NR, LV, Fig. 1B), to locally extensive or disseminated masses deforming facial structures (e.g. RV, SN, GV, Fig. 1B). Metastases are common in DFTD (5), and were found in submandibular lymph node (SMLN) in NS,
and in SMLN, lung and kidney in RV. Gross features of tumours in these animals were suggestive and typical of DFTD associated with the first described DFTD clone, DFT1.

Initial histological assessment of two of the Channel DFTD cases from 2014, RV and SN, revealed that tumours from these animals presented atypical features. DFTD tumours associated with the DFT1 clone are generally composed of pleomorphic round cells arranged in distinct bundles, cords or packets (5). In contrast, the tumours from RV and SN were characterised by sheets of pleomorphic (amorphic to stellate and fusiform) cells arranged in a solid pattern (Fig. 2). Furthermore, tumours from RV and SN were negative for periaxin (PRX), an immunohistochemical marker that is diagnostic for DFT1 (12, 17) (Fig. 2). Although it was initially considered plausible that tumours from RV and SN were DFT1 variants or spontaneous tumours arising from the tissues of their hosts, further cytogenetic and genetic analyses were performed to confirm the nature of these two tumours and other tumours derived from devils in the Channel Peninsula.

Cytogenetic profile of DFT2

The clonal nature of DFTD was first suggested based on cytogenetic evidence indicating that DFTD tumours carry a distinctive aneuploid karyotype (9). This DFT1 cytogenetic profile differs markedly from the normal devil karyotype, and is characterised by the absence of identifiable chromosome 2 homologues, the presence of four marker chromosomes and missing sex chromosomes (Fig. 3). However, cytogenetic analysis of samples from the Channel revealed that tumours from five of the devils (those derived from RV, SN, JV, NR and NS) shared an identical aneuploid karyotype that was clearly distinct from that of DFT1 (Fig. 3; data from NS not shown). Chromosomes from these tumours all exhibited identical complex structural abnormalities, including the presence of additional material on chromosomes 1, 2 and 4, a deletion involving chromosome 5 and monosomy for chromosome 6. Both X and Y sex chromosomes were present. This cytogenetic evidence presented the possibility that these five tumours were derived from a clone, which we have named DFT2, that is distinct from DFT1.

Genetic analysis of DFT2

Despite the absence of cytogenetically identifiable sex chromosomes in DFT1, studies using fluorescence in situ hybridisation and DNA sequencing have found evidence for two X chromosome copies in DFT1 (13, 14). Furthermore, the number of single point substitution variants mapping to the X chromosome in DFT1 suggested that the two X chromosomes were germline homologues rather than recent somatic duplicates (13). We further investigated the origins of the X chromosomes in DFT1 by genotyping a panel of ten X chromosome variants that were heterozygous in DFT1 in a group of male and female devils. At each locus, we found that both alleles could be detected in the devil population, confirming that these variants are germline single nucleotide polymorphisms (SNPs) rather than somatic mutations (Fig. 4A). Furthermore, our analysis confirmed that these SNPs indeed map to the X chromosome as only females, and not males, were found to be heterozygous at these loci (Fig. 4A). These data confirm that DFT1 carries DNA from two homologous X chromosomes, indicating that this lineage probably arose in a female devil. The discovery that DFT2 carries a Y chromosome is thus incompatible with a single clonal origin for DFT1 and DFT2.

To investigate the genetic relationship between DFT1 and DFT2, we analysed the genotypes of these two lineages at nine polymorphic microsatellite loci (10, 12, 18, 19). Our analysis confirmed that two DFT1 tumours, 87T and 88T, shared an identical genotype with each other and with a previously analysed panel of 27 DFT1 tumours collected from geographically dispersed areas of Tasmania (12) (Fig. 4B, Table S2). Analysis of DFT2, however, revealed that the tumours derived from RV and SN had different genotypes from DFT1 (Fig. 4B). Furthermore, these two tumours shared identical genotypes with each other and were genetically distinct from their hosts (Fig. 4B). Indeed, the microsatellite analysis indicated that DFT2 tumours were no more similar to DFT1 (identical genotypes at two of nine loci) than they were to their hosts (identical genotypes at four
and three of nine loci for RV and SN respectively), or to other devils in the population (e.g. 53H, a devil from northern Tasmania) (Fig. 4B).

We further characterised DFT1 and DFT2 by analysing panels of tumours for the presence of twelve putative somatic structural variants that had previously been identified in DFT1 (13), as well as for the presence of two polymorphic germline structural variants (Fig. 4C). None of the twelve putative somatic structural variants were found in DFT2 tumours, although they were found in DFT1 tumours 87T and 88T (Fig. 4C). One of the polymorphic germline structural variants was present in DFT2, but was absent in DFT1 and host tissue derived from RV (Fig. 4C).

DFTD tumours are allogeneic grafts within their hosts. Previous studies have confirmed that DFT1 is able to colonise hosts carrying different genotypes at MHC loci (10, 20). However, MHC molecules are not expressed on the cell surface of most DFT1 cells, and this has been proposed as a mechanism whereby this lineage escapes destruction by host T cells (11). To investigate whether DFT2 is able to grow in hosts with disparate MHC genotype, we cloned and sequenced part of the polymorphic second exon of MHC class I loci from two DFT2 tumours and their corresponding hosts. We identified five MHC class I exon 2 haplotypes in DFT2, and confirmed that DFT2 has a different MHC class I genotype from DFT1 (Fig. 4D). Importantly, the MHC class I genotype found in DFT2 was distinct from the genotypes found in DFT2 hosts, confirming that DFT2 is not restricted to hosts with an identical MHC class I genotype (Fig. 4D).

DISCUSSION

The data presented here indicate that DFT2 is a transmissible cancer that is distinct from DFT1. Although DFT2 has so far been detected in only five male devils located on the Channel Peninsula in south-east Tasmania in 2014 and 2015, the extent of its current distribution in the devil population, and its location and time of origin, remain unknown.

Our analysis suggests that DFT2 may have arisen as a second independent transmissible cancer in Tasmanian devils manifesting as facial tumours that are outwardly indistinguishable from those caused by DFT1. This finding would challenge our current understanding that the emergence of transmissibility in cancer is an extraordinarily rare occurrence in nature. Thus it is possible that the burden of transmissible cancers as pathogens in natural populations has been underestimated; alternatively, transmissible cancers may emerge rarely in most species, but species-specific vulnerabilities may promote their emergence within certain host populations.

The discovery of a second transmissible cancer in Tasmanian devils suggests that this species may be particularly at risk for the emergence of transmissible cancers. This could perhaps be mediated by this species’ apparent elevated susceptibility to neoplasia (21, 22), low genetic diversity (10, 23-25) and/or biting behaviour (26). However, if this is the case, it is surprising that tumours comparable with DFTD were not reported prior to 1996 (4, 5). It is possible, however, that additional Tasmanian devil transmissible cancers may currently exist, or previously occurred, but have remained undetected. It is also possible that exposure to novel pathogens or anthropogenic factors may have influenced the propensity of Tasmanian devils to develop transmissible cancers. The potential for new transmissible cancer clones to emerge in this species has important implications for Tasmanian devil conservation programmes, whose success largely depend on the long-term viability of isolated captive insurance populations.

Tasmanian devils have low levels of genetic diversity (10, 23-25), possibly caused by historical population declines driven by past climate change (27). Remaining genetic diversity was possibly further eroded by persecution following European settlement of Tasmania, and, more recently, by the DFTD epidemic (23, 27). Low genetic diversity may have contributed to risk for the emergence of transmissible cancers in this species.
The possibility that clonally transmissible cancers may arise more frequently in nature than previously considered warrants further investigation of the risk that such diseases could arise in humans. Although transfer of cancer cells between two humans has been reported in rare circumstances, involving injury, organ transplantation, experimental treatments or pregnancy (3), no human cancer has been observed to naturally transmit between more than two human hosts.

An alternative explanation for the existence of two distinct transmissible cancer clones in Tasmanian devils is the possibility that DFT1 and/or DFT2 arose via hybridisation of genetic material derived from ancestral DFT cells and host cells. Horizontal transfer of mitochondrial DNA between host cells and cancer cells has been documented in CTVT (28), indicating that, at least in CTVT, there are mechanisms that permit exchange of genetic material between host cells and cancer cells. However, our failure to find any significant evidence for shared DNA markers between DFT1 and DFT2 suggests that, if horizontal transfer has occurred, then host cell DNA largely replaced the ancestral DFT DNA in at least one of DFT1 or DFT2. If this was the case, then DFTD may provide insight into somatic cell hybridisation in cancer, a phenomenon that may be challenging to detect in cancers that remain in one host (29, 30).

The discovery of a second transmissible cancer in Tasmanian devils changes our perception of the potential of cancer cells to adapt to new niches as parasitic clonal cell lineages. Regardless of whether the plight of Tasmanian devils represents the existence of a common pathological process that has previously been overlooked, is the consequence of an unfortunate species-specific vulnerability, or has arisen due to an exceptionally improbable concomitance of events, clarification of the biological basis of DFT2 promises to illuminate important concepts underpinning cancer evolution.
METHODS

Animals
Wild Tasmanian devils in the Channel area with signs of DFTD were either trapped or found dead from road trauma or other causes. Live devils with visible signs of distress were euthanised for welfare reasons. Tissue biopsies and tumour fine needle aspirates were either collected post mortem, or from live devils which were subsequently released. All animal procedures were performed under a Standard Operating Procedure approved by the General Manager, Natural and Cultural Heritage Division, Tasmanian Government Department of Primary Industries, Parks, Water and the Environment (DPIPWE), in agreement with the Animal Ethics Committee.

Histology and immunohistochemistry
Standard haematoxylin and eosin (H&E) and immunohistochemical staining with a periaxin antibody (Sigma Aldrich, HPA001868) were performed on three-micrometre paraffin sections from tumour tissues fixed in 10% neutral buffered formalin as previously described (17). Histological sections were not available for JV or NR, and data from NS are not shown.

Cytogenetics
Karyotyping was performed as previously described (31). Chromosomes were banded with Leishman’s stain (Sigma-Aldrich) according to standard methods. Analysis and imaging were performed using Metasystems™ karyotyping software. Karyotyping for NS is not shown.

X chromosome analysis
Candidate SNPs mapping to the X chromosome that were heterozygous in DFT1 tumours as well as in the germline of a female devil (91H) were identified from whole genome sequence reads (13). Ten of these SNPs were genotyped in 74 devils (37 males and 37 females), as well as in ten DFT1 tumours and two DFT2 tumours, by PCR and capillary sequencing; DNA was only available for two DFT2 tumours (those from RV and SN) during preparation of the manuscript. Tables S3 and S4 have details of PCR primers, samples and genotypes. All PCRs were performed under standard conditions with annealing temperature of 60°C, extension time of 45 seconds and 31 cycles.

Microsatellite genotyping
Microsatellite genotyping was performed as previously described (12). Briefly, PCR was performed with primers listed in Table S2; each forward primer had a 19 bp M13F sequence (5’-AGG-AAA-CAG-CTA-TGA-CCA-T-3’) attached to its 5’ end. PCR was performed with 57°C annealing temperature, reducing the annealing temperature by 1°C per cycle for 6 cycles, followed by 31 cycles with annealing temperature 50°C; at this step, 8 pmol of 5’-6FAM or 5’-HEX labelled M13F primer was added to each reaction. The reaction was then continued for 8 additional cycles with annealing temperature 48°C. Products were separated on an ABI 3730XL instrument and analysed using GeneMarker software. Concordance of alleles shown in Fig. 4B with previously published microsatellite alleles (12) is provided in Table S2.

Structural variant analysis
Whole genome sequence reads derived from two DFT1 tumours and a male and female devil (31H and 91H) were analysed using an algorithm that uses discordantly mapped read pairs to identify putative structural variants, as previously described (13). A set of fourteen putative structural variants were analysed by PCR with DNA from DFT1 and DFT2 tumours and as well as with germline DNA from devils. PCRs were performed under standard conditions with annealing temperature of 60°C and 35 cycles. Primer sequences can be found in Table S5.

MHC analysis
A 274 bp DNA segment was amplified from genomic DNA using primers recognising the polymorphic exon 2 of devil MHC class I loci. Primer sequences, Sahaα1F (5’-TCT CAC TCC TTG AGG TAC TTC G-3’) and Sahaα1R2 (5’-CTC GCT CTG GTT GTA GTA GCC G-3’) were modified
from (32); modifications to Sahaα1R (32) were made based on sequence alignments available in (25). The number of loci amplified by these primers is not known, and may vary between individuals, but is predicted to be between four and five (33, 34); furthermore, we cannot exclude the possibility that these primer sequences select for only a subset of haplotypes due to polymorphisms present within primer binding sites. Libraries were prepared from amplicons from each individual and sequenced on the Illumina MiSeq platform with 150 bp paired end reads. Between 100,000 and 900,000 read pairs were sequenced per individual. Forward and reverse sequences were merged for each read pair, discarding any read pairs with mismatches in the overlapping region as well as any read pair which did not exactly match both primer sequences, yielding between 10,000 and 23,000 unique haplotypes per individual. Of these, only 17 haplotypes which were present with at least one per cent frequency in at least one individual were considered further. We further validated these haplotypes by searching for reads exactly matching the haplotypes within the available whole genome sequences from 87T and 91H (13). One haplotype could not be validated using this approach and was discarded. The sequence composition of two further haplotypes meant that they could only be validated in conjunction with another haplotype, and the pattern of haplotype read counts made it uncertain if these two haplotypes were present in some individuals; thus these two haplotypes were excluded from Fig. 4D. Table S6 contains nucleotide and predicted amino acid sequences for the MHC class I haplotypes presented in Fig. 4D. Each of the fourteen haplotypes in Fig. 4D are predicted to encode a unique peptide sequence. Two haplotypes that had not previously been described were named SahaI*97 and SahaI*98 and their sequences submitted to Genbank with accession numbers KT188437 and KT188438 respectively.
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REFERENCES


FIGURE LEGENDS

Fig. 1. Geographical location and gross appearance of DFT2 tumours.
(A) Locations of confirmed DFT1 and DFT2 tumours in Tasmania (left) and the Channel Peninsula (right). Each DFT1 location is represented with a single dot regardless of the number of tumours identified at this location. Tumour diagnosis was performed by histopathology, cytogenetics and/or genetic analysis.
(B) Gross appearance of two DFT1 tumours (left) and four DFT2 tumours (right). Tumours were identified in Tasmanian devils in the Channel region between 2012 and 2015. Further information about animals is available in Table S1.

Fig. 2. DFT2 tumours are histologically distinct from DFT1.
Representative images of haematoxylin and eosin (H&E) stained histological sections of DFT1 and DFT2 tumours (upper and middle panel). Lower panel, histological sections stained with DFT1 marker, PRX. Scale bars represent 200 µm (upper panel) or 100 µm (middle panel and lower panel). Arrows indicate peripheral nerve bundles, which are positive for PRX.

Fig. 3. DFT2 tumours are cytogenetically distinct from DFT1.
Representative karyotypes of a normal male devil, a DFT1 tumour and four DFT2 tumours. Red arrows indicate chromosomes carrying cytogenetic abnormalities. Four marker chromosomes found in DFT1 (9) are labelled M1 to M4.

Fig. 4. DFT2 tumours are genetically distinct from DFT1 and from their hosts.
(A) Genotyping of X-linked SNPs. Genotypes of 37 female and 37 male devils at 10 X-linked SNP loci that are heterozygous in DFT1. Both homozygous and hemizygous genotypes are referred to as “homozygous”. Each individual is represented by a column, and chromosome and scaffold for each locus is shown. Further information about individuals and exact SNP coordinates are available in Tables S3 and S4.
(B) Microsatellite genotypes at nine polymorphic microsatellite loci (L, E, D, N, C, M, J, F and K). The lengths of the two (L, D, N, M, J, F and K) or three (E and C) alleles found at each locus and their colour codes are shown on the left, and their genotypes in DFT1 tumours, DFT2 tumours, DFT2 hosts and in 53H, a representative unrelated devil, are shown. All tumours are presented as diploid, although true copy number at these loci is not known. Further information about allele sizes is found in Table S2.
(C) Structural variant genotyping. PCRs spanning breakpoint junctions were performed to assess the presence or absence of twelve possibly somatic structural variants and two polymorphic germline variants. We cannot confirm if structural variants found only in DFT1 are somatic or rare germline variants, thus these are labelled “potentially somatic”. Chromosomes and scaffolds involved in each rearrangement are indicated, and full breakpoint coordinates are available in Table S5.
(D) MHC class I exon 2 haplotypes detected in DFT1 tumours, DFT2 tumours, DFT2 hosts and in 91H, a representative unrelated devil. Exon 2 haplotype names are indicated on the left, and their presence or absence in the panel of samples are indicated with blue and white squares respectively. All fourteen haplotypes are predicted to encode a unique amino acid sequence. Complete sequences for each haplotype are available in Table S6.
SUPPLEMENTARY INFORMATION

Table S1.
DFTD in the Channel area. Details of the twelve confirmed DFTD cases (both DFT1 and DFT2) in the Channel area from the first reported case in December 2012 until June 2015. Date indicates the date when samples were collected from the animal, and age is the estimated age of the animal on that date.

Table S2.
Microsatellite primers and allele information. Concordance between alleles reported in a previous study (12) and those from the current study is indicated.

Table S3.
Genotype and sample summary for X chromosome SNP analysis.

Table S4.
Genome coordinates and primers for X chromosome SNP analysis.

Table S5.
Structural variant breakpoint coordinates and primers.

Table S6.
MHC class I exon 2 haplotypes. Nucleotide and amino acid sequences for fourteen MHC class I (SahaI) exon 2 haplotypes included in Fig. 4D.
A

Females

Males

DFT1

DFT2

DFT2 hosts

Unrelated devil

B

Alleles

DFT1

DFT2

DFT2 hosts

Unrelated devil

C

D

Sahal*32
Sahal*88
Sahal*29
Sahal*35
Sahal*36
Sahal*33
Sahal*97
Sahal*46
Sahal*27
Sahal*90
Sahal*98
Sahal*37
Sahal*49
Sahal*45

Homozygous (REF)  Homozygous (ALT)  Heterozygous

Chr2:GL841444 - Chr2:GL841242
Chr2:GL841455 - Chr2:GL841454
ChrX:GL867598 - Chr2:GL841420
Chr5:GL861565 - Chr5:GL861655
Chr5:GL861665 - Chr2:GL841451
Chr5:GL861708 - Chr3:GL849528
Chr1:GL834489 - Chr1:GL834489
Chr4:GL857677 - Chr1:GL834488
Chr2:GL841709 - Chr2:GL841454
ChrX:GL867695 - ChrX:GL867579
Chr3:GL849903 - Chr3:GL849903
Chr5:GL861537 - Chr2:GL841419
Chr1:GL834551 - Chr1:GL834446
Chr3:GL849558 - Chr3:GL849558