RESEARCH NOTE

No evidence for clonal transmission of urogenital carcinoma in California sea lions (*Zalophus californianus*) [version 1; referees: 3 approved]

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Abstract

Urogenital carcinoma is a highly metastatic cancer affecting California sea lions (*Zalophus californianus*). The disease has high prevalence amongst stranded animals, and is one of the most commonly observed cancers in wildlife. The genital localisation of primary tumours suggests the possibility that coital transmission of an infectious agent could underlie this disease. Otarine herpesvirus type 1 has been associated with lesions, however a causative role for this virus has not been confirmed. We investigated the possibility that urogenital carcinoma might be clonally transmissible, spread by the direct transfer of cancer cells. Analysis of sequences at the mitochondrial DNA control region in seven matched tumour and host pairs confirmed that tumour genotypes were identical to those of their matched hosts and did not show similarity with tumours from other individuals. Thus our findings suggest that urogenital carcinoma in California sea lions is not clonally transmitted, but rather arises from transformed host cells.

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version 1 published 22 Jun 2017	✓ report	✓ report	✓ report

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Comments (0)

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Introduction

Urogenital carcinoma (UGC) is the most commonly observed neoplasm in California sea lions (*Zalophus californianus*)[1]. This cancer was first reported in sea lions on the west coast of North America in 1979[2], and over a fifteen-year period, from 1998 to 2012, the disease was found in 26 per cent of adult animals examined post-mortem at The Marine Mammal Center, California[1]. UGC affects both male and female animals, and is most frequently found in sexually mature adults[3,4]. The disease typically presents with extensive multi-organ metastases; however, primary lesions involving the genital epithelium can usually be identified[5].

Three aetiological factors have been proposed for the development of UGC: infection, host genetics, and environmental factors. Otarine herpesvirus type 1 (OtHV-1), a gammaherpesvirus related to Kaposi's sarcoma-linked human herpesvirus-8[5,6] has been associated with UGC[5–7]; however, this virus has not been confirmed as a causative agent. An association between UGC and genital bacterial infection has also been proposed[8]. Genetic studies have indicated that individuals with high parental relatedness[9], homozygosity at the *HSPE2* locus[10], or one or more copies of the *Zaca-DRB.A* MHC class II locus[11] have increased risk of UGC. Environmental contaminants, such as organochlorines, have also been proposed as causative agents in UGC carcinogenesis[12].

Cancer occurs when a somatic cell acquires mutations that drive it towards a program of uncontrolled clonal expansion. Although cancer cells can migrate and invade distant tissues, most cancers remain within the body of the host that spawned them. Rarely, however, cancers can become transmissible such that cancer cells themselves become infectious agents that are transferred between individuals as allogeneic grafts. Only eight examples of naturally occurring contagious cancers are known: canine transmissible venereal tumour (CTVT) found in domestic dogs[13,14], two distinct lineages of Tasmanian devil facial tumour disease[15,16], and five lineages of disseminated neoplasia affecting various species of marine bivalves[17,18]. Tumours derived from clonally transmissible cancers carry the genetic material of the original animal that first gave rise to the cancer; thus, transmissible cancers are characterised by shared genotypes that are distinct from those of their matched hosts.

Several features of UGC are compatible with the possibility that this cancer is clonally transmissible: epidemiological observations of UGC are consistent with an infectious aetiology for the disease[2]; and, in particular, its genital localisation could provide a coital route of transmission[19], as is observed with CTVT, the transmissible cancer in dogs.

We genotyped UGC tumours and their matched hosts to determine if UGC is clonally transmissible. Our results do not show evidence for UGC being a transmissible cancer, but rather confirm that UGC tumours are most likely derived from their hosts.

Methods

Ethics

This study was approved by The Marine Mammal Center Institutional Animal Care and Use Committee (Sausalito, CA) and the National Marine Fisheries Service MMPA (permit number 18786).

Samples

Tissues from seven wild stranded adult California sea lions were collected at The Marine Mammal Center, Sausalito, CA. Complete gross and histopathological examinations were performed on each animal to confirm UGC diagnosis. Tumour (metastasis) and host tissue (liver or muscle) biopsies were collected into RNAlater during post-mortem examination and were stored at −70°C until processing.

DNA extraction

Representative tissue sampled from tumour and host biopsies was used for DNA extraction using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. DNA was quantified using a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA).

PCR

We amplified a 1289 bp fragment of the mitochondrial DNA (mtDNA) control region using primers described by Wolf *et al*[20]. PCR was performed using an Eppendorf Mastercycler Nexus GSX1 (Eppendorf, Hamburg, Germany) with conditions as follows: 40 ng of genomic DNA was amplified in a total volume of 20 µl containing 0.5 µM of each primer, 0.2 mM of each dNTP and 0.02 units of Taq DNA polymerase (Qiagen, Hilden, Germany) per reaction. Cycling conditions were 95°C for 3 min, 30 cycles of 95°C for 15 s, 60°C for 30 s, 72°C for 45 s and a final extension step at 72°C for 5 min. PCR products were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany). Purified PCR products were capillary sequenced at Source BioScience LifeSciences Genomic Services (Source BioScience LifeSciences, Nottingham, United Kingdom).

Alignment and variant calling

Sequences were aligned to the California sea lion mtDNA reference genome (accession number NC_008416)[21] using Sequencher DNA Sequence Analysis Software v5.4.6 (Gene Codes, Ann Arbor, MI, USA). Alignment errors were inspected manually and corrected. Variant positions were identified by viewing alignments, as well as by manual assessment of sequence chromatograms using FinchTV v1.4.0 (Geospiza Inc., Seattle, WA, USA). Variants were only assessed within a 397 bp region of the product, comprising region 15490–15886 in NC_008416.

Results

We assessed 397 base pairs of the mtDNA control region in seven UGC tumours and their matched hosts. The analysis identified nine polymorphic sites characterising four unique genotypes within the sampled sea lion population (Table 1). In all cases, the alleles present in tumours were identical to those found in matched host tissue (Table 1). Chromatograms were closely examined at

Table 1. Mitochondrial DNA (mtDNA) genotypes at nine polymorphic sites in sea lion hosts and matched tumours. Coordinates are relative to the sea lion mtDNA reference genome, NC_008416[21]. Individual California sea lions (CSLs) are labelled numerically and matched hosts and tumours are represented side-by-side. Alleles, represented by nucleotide code (A,C,G,T), are shown. Alleles that differ from the reference are shaded in grey.

	Individual:	Reference	CSL 1		CSL 2		CSL 3		CSL 4		CSL 5		CSL 6		CSL 7	
	Tissue:		Host	Tumour	Host	Tumour	Host	Tumour	Host	Tumour	Host	Tumour	Host	Tumour	Host	Tumour
mtDNA coordinate	15524	T	T	T	T	T	T	T	T	T	T	T	C	C	T	T
	15527	T	T	T	T	T	T	T	C	C	C	C	C	C	T	T
	15528	T	T	T	T	T	T	T	C	C	C	C	C	C	T	T
	15550	G	G	G	G	G	G	G	A	A	A	A	A	A	G	G
	15551	A	A	A	A	A	A	A	G	G	G	G	A	A	A	A
	15629	C	C	C	C	C	C	C	C	C	C	C	C	C	T	T
	15652	A	A	A	A	A	A	A	A	A	A	A	G	G	A	A
	15660	T	T	T	T	T	T	T	C	C	C	C	C	C	T	T
	15812	G	G	G	G	G	G	G	A	A	A	A	A	A	G	G

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polymorphic sites, but no evidence for amplification of additional alleles in tumour tissues was found[22].

Discussion

Our study does not support the hypothesis that UGC is clonally transmitted, but rather further confirms that UGC arises from host cells. Importantly, however, we cannot exclude the possibility that some UGCs are clonally transmitted. Given the genital localisation of this cancer, and likely accessibility of UGC cancer cells to other individuals during coitus, UGC tumours may pose a particular risk for the emergence of a transmissible cancer clone.

In this analysis we only examined genetic variation at one mtDNA locus. It is worth noting that at least one transmissible cancer – CTVT in dogs - has been observed to periodically capture mtDNA from its hosts[23]; thus, mtDNA may not be considered the most reliable marker for testing clonality in transmissible cancers. However, mtDNA horizontal transfer events were detected only five times in a cohort of 449 CTVT tumours[24]; thus even if mtDNA capture had occurred, it would not be expected that tumours would genetically match their hosts as frequently as we have observed in UGC.

Given that transmissible cancers are clonal lineages, tumour cell morphology and tissue architecture is generally very similar between tumours[25,26]. However, previous research has shown that UGCs appear to develop through histologically distinct stages[5], which further supports the idea of step-wise oncogenic transformation of host tissue rather than direct transmission of a cancer lineage.

Future research exploring the role of viral agents, host genetics and environmental factors, as well as somatic genetics, will be important for understanding the carcinogenic processes that cause UGC. It is interesting to note that an OtHV-1-associated UGC has recently been reported in a South American fur seal (*Arctocephalus australis*)[27], indicating that other pinnipeds are susceptible to UGC, and further implicating OtHV-1 as a causative agent. Furthermore, a recent analysis of cytological smears collected from California sea lions in the Gulf of California revealed that transformation of the genital epithelium may be relatively common in this species[28].

Wildlife models of cancer can provide novel insights into general mechanisms of cancer development[29]. Furthermore, an understanding of the aetiological factors underlying commonly observed cancers in wildlife is important for conservation and biomonitoring. In this study, we have found no evidence that UGC, one of the few "cancer epidemics" in wildlife[1,29], is clonally transmitted. Ruling out this mode of carcinogenesis is an important step in our understanding of UGC, and paves the way towards further research investigating the processes underlying this aggressive disease in sea lions.

Data availability

Data associated with this study are available in GenBank with accession numbers: MF000998 - MF001011.

Author contributions

MNL and EPM conceived the study. FMD contributed unpublished essential data. MNL carried out the analysis. MNL and EPM prepared the first draft of the manuscript. All authors were involved in the revision of the draft manuscript and have agreed to the final content.

Competing interests

No competing interests were disclosed.

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Current Referee Status: ✔ ✔ ✔

Version 1

Referee Report 17 July 2017

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✔ **Beata Ujvari**
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Although many cancers are associated with infectious agents[1], only a few naturally occurring transmissible cancers have so far been identified in dogs, soft-shell clams and in Tasmanian devils. The lack of evidence for transmissible cancers can be related to several factors: (i) although neoplasia has been recorded in most metazoans, wildlife cancer statistics are, however, highly scattered in the scientific literature and hence challenging to access[2], (ii) lack of systematic screening (using large datasets) for the presence of clonally transmitted malignant cells in wildlife, (iii) failure to recognize extinct contagious cancers (that have been eliminated due to their detrimental impact on host fitness during the eons of evolution). Similar to pathogens/parasites, neoplasia (particularly communicable ones) can have a significant negative impact on host fitness in the wild and therefore be an important, but so far overlooked, evolutionary force that should not be ignored. The study by Ní Leathlobhair *et al*. is one of the first, hopefully many more to come, systematic studies that attempts to identify the etiology of a wildlife cancer with high prevalence. The authors should be recommended for publishing negative results in search of wildlife transmissible cancers.

In general, the paper would have benefited from higher sample sizes and the inclusion of more molecular markers into the analyses, although most likely the outcome of the paper, refuting the transmission hypothesis, would not have changed.

One general concern with the article is basing the analysis solely on a fragment of mtDNA without acknowledging the potential hazard of amplifying nuclear mitochondrial DNA segments (NUMTs). NUMTs are insertions of mitochondrial DNA sequences into the nuclear genome, becoming pseudogenes, which are thought to be the remnants of gene transfer from mitochondria to nucleus[3]. The transposition of mtDNA sequences into the nuclear genome has been documented in a wide variety of taxa, from fungi to insects to vertebrates[4]. Although mounting evidence is suggesting that the occurrence of NUMTs is a ubiquitous phenomenon[5] studies frequently overlook their potential presence and generate unreliable results. NUMTs can be amplified in PCR reactions alongside their mitochondrial counterparts, becoming a source of contamination in mtDNA analysis[6]. This is an issue that is often overlooked in studies of mtDNA and can result in misguided conclusions. Although the outcome of the study by Ní Leathlobhair *et al.* would not have changed by the presence of NUMts, it would be so nice to see the scientific community finally acknowledging that simply amplifying fragments of mtDNA is not an acceptable scientific method to conduct evolutionary, population genetics, phylogenetic etc. studies. There are several practices to screen for the presence of NUMTs, e.g. serial dilution method [7], nested approach to the PCR reactions[8],

isolation of the mitochondrial genome during DNA extraction [9], digestion of nuclear DNA with DNase[10], sequencing of the entire mitochondria genome, for example through next generation sequencing (but see Verscheure, et al., 2015[11], also Calvignac, S. et al. 2011[12], Hazkani-Covo, E. et al. 2010[13]).

In summary, the study by Ní Leathlobhair *et al*. is a nice contribution to the transmissible cancer literature, a delight to read, but I would highly encourage the authors of any future population genetic, phylogenetic and evolutionary studies to refrain from only analysing fragments of mtDNA, failing to amplify pure mtDNA can lead to unreliable results and erroneous conclusions. I would really like the practice of mtDNA fragment analysis (without eliminating NUMTs) to disappear from future scientific works.

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Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Referee Report 06 July 2017

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Nicolas Bierne
CNRSUMIRD (French National Center for Scientific Research-UM- Institute of Research for Development), Institute of Evolutionary Science of Montpellier (ISEM), Montpellier, France

Leathlobhair *et al.* conducted a straightforward test of the hypothesis that sea lions urogenital carcinoma (UGC) could be a transmissible cancer. They sequenced the mtDNA control region of seven tumors and their respective host tissue. They observed four different haplotypes in their sample and in each of the seven comparisons the two sequences were identical. This was the case even after a close inspection of the chromatograms to verify the presence of a possibly under-amplified sequence. Leathlobhair *et al.* study therefore does not support the hypothesis that sea lions UGC is a clonally transmitted cancer.

Transmissible cancer is thought a rare exception. However, this conclusion suffers from a lack of a rigorous appreciation of the effort invested to test for genetic differences between tumor and host cells. In the wildlife especially, it is difficult to know how often the hypothesis of transmissible cancer is tested. For this reason the publication of negative results is essential and needs to be promoted. Although sea lions UGC was a pertinent suspect with an infectious aetiology suspected for years and a possible route of transmission via sexual relationship as in CTVT, the hypothesis of clonal transmission has not been tested/published before the timely study of Leathlobhair *et al.*

The paper is clearly written and addresses all the relevant issues. I would suggest the authors to take the opportunity of their publication to promote a more systematic examination of the hypothesis of cancer transmission, possibly with an associated database. One question raised by Leathlobhair *et al.* study is about the genotyping effort required to definitively reject direct infection by cancer cells. Here the authors analyzed only 7 pairs with only one mtDNA marker with low nucleotide diversity. They rightly discussed the issue of mitochondrial captures, already reported in CTVT, which would be required to explain their data under the transmission hypothesis. However, only three captures is required to generate the four

haplotypes observed in tumors. What makes the transmission hypothesis unlikely is the perfect match observed for rare haplotypes in CSL 6 and CSL 7. Nonetheless the authors could have provided a better idea of the nucleotide diversity observed in a wider sample of sea lions by using published data (eg in Gonzalez-Suarez *et al.* 2009 Mol Ecol, Schramm *et al.* 2009 Mar Biol). In addition, horizontal transfer has been found rare in the two mammalian transmissible cancers found to date, but this can be different for another new one. Riquet *et al* (2017) found 5 chimeric mussels thought to be infected by transmissible neoplasia with a combination of a nuclear SNP array and mtDNA sequences, but only two had the mtDNA of the transmissible lineage initially found in the pacific mussels by Metzger *et al.* (2016). The other three individuals had a perfect match with their host with mtDNA sequences while being chimeric at many nuclear SNPs. With only those three mussels and the use of mtDNA sequences, they would have missed the hypothesis of transmissible cancer in their sample. I would therefore suggest the authors to encourage the use of more markers, even if I am convinced that this first screen mostly refutes the transmission hypothesis. Genotyping a handful of nuclear markers is easy nowadays and I would not like subsequent studies to content to use only mtDNA sequences. Finally, one can suspect not so unlikely that standard oncogenic transformation of host tissue can sometimes infect a new host with few success of subsequent propagation -e.g. killing the new host rapidly because already at the metastatic stage. We would have a combination of standard and transmissible cancer lineages that would need a more extensive examination.

To conclude, I very much enjoyed this paper, the approach is straightforward, I think a test of genetic chimerism in such a serious candidate as sea lions UGC was more than timely, I mostly concur that the hypothesis of clonal transmission can be rejected, and I thank the authors to promote by this submission a systematic search of transmissible cancer in the wildlife and the publication of “negative” results.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Referee Expertise: Population genetics

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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The Ní Leathlobhair *et al.* paper is an interesting article investigating the etiology of a cancer with high prevalence in California sea lions, specifically testing the hypothesis that it could be due to a transmissible cancer. Findings of transmissible cancer in multiple species has raised the possibility that other cancers could be due to transmission of cancer cells, specifically those that occur at high prevalence and those that have a plausible route of transmission, as are the case with the sea lion urogenital carcinomas.

The finding of mtDNA SNPs between individuals, but no evidence of a neoplasia-associated allele strongly suggests that this disease is not a transmissible cancer. Since this result relies on the lack of detection of additional alleles on the chromatograms at polymorphic sites, it would be good to see at least a few examples of images from the chromatograms. However, given the analysis of paired host/tumor samples, this should have been obvious if present, unless the tumor cells only represent a small fraction of the tumor sample. Nuclear loci could have been checked to confirm this finding, but, as stated in the paper, in order to have transmissible cancer with no differences in the mtDNA it would require an unprecedented amount of mitochondrial transfer.

Minor issue: It is unclear what primers were used for the PCR. The methods says that a 1289 bp fragment was amplified, but the reference cited appears to use two primer pairs to amplify mtDNA, generating 625 and 500 bp products. This reference (Wolf *et al.*) includes a table of other primers, but doesn't appear to list the primers for these PCR reactions, but the Features sections in the GenBank submissions reported in the paper does. Since neither of the primer pairs reported in the Wolf *et al.* study match the size reported, it is unclear if these are the primers used, however. It should be simple to add the sequences of the primers used to clarify the methods, as there was only one PCR done in this study.

Overall, the results were negative, but it is important and valuable to other researchers to publish them, as it is a plausible hypothesis to explain the etiology of this common cancer.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

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