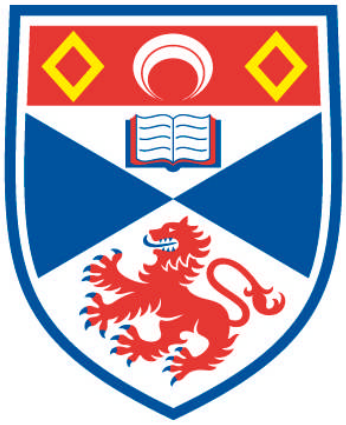
THE MULTI-FACTORIAL AETIOLOGY OF UROGENITAL CARCINOMA IN THE CALIFORNIA SEA LION (ZALOPHUS CALIFORNIANUS) – A CASE-CONTROL STUDY

Helen M. Browning

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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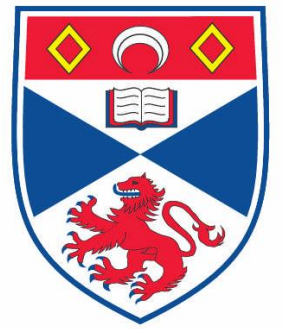

This thesis is submitted in partial fulfilment for the degree of PhD
at the
University of St Andrews

April 2014

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Abstract

California sea lions (CSLs) have an unusually high occurrence of urogenital cancer (UGC), with studies revealing metastatic carcinoma in 26 % of CSLs admitted to a rehabilitation centre between 1998 and 2012. It is likely that the aetiology of this disease is multi-factorial as genetics, viral infection and exposure to contaminants have been associated with this cancer to date. The goal of this study was to investigate the association of a number of factors using a case-control study design on animals admitted to a rehabilitation centre. The study additionally concentrates on two main areas; (i) genetic factors and (ii) the presence of herpesvirus.

Previous investigations identified cancer to be more likely in animals with specific microsatellite alleles. In the present study genotyping of CSLs at three microsatellite loci revealed that homozygosity at one marker (Pv11) was significantly associated with the presence of the disease. Pv11 was found to be located within a gene called heparanase 2 (HPSE2) and investigations into the expression of its protein revealed differences according to Pv11 genotype.

The presence of herpesvirus was investigated by two PCR methods and identified the gammaherpesvirus OtHV-1. The results of the two methods were contradictory with one method identifying a highly significant relationship between the presence of OtHV-1 and UGC whereas the other did not. Complicating factors such as potential differences in sensitivity of the tests along with the possible presence of closely related viruses or variants of OtHV-1 may explain this.

The availability of necropsy data for the CSLs in the study allowed the inclusion of body condition data in the statistical analysis to evaluate other potential risk factors. Final analysis revealed the presence of three risk factors; Pv11 genotype, OtHV-1 presence and thinner blubber.

This study is the largest study undertaken so far in order to investigate the involvement of risk factors associated with UGC in the CSL and supports a multi-factorial aetiology of this disease.

Acknowledgements

Firstly I would like to thank my supervisors Ailsa Hall and John Hammond for all their help and encouragement throughout this project. I am extremely grateful for their endless support especially in always making themselves available whenever I required guidance.

I would like to thank the staff of The Marine Mammal Center, particularly Frances Gulland without whom this project would not have been possible. Also Denise Greig and Lauren Rust for answering my numerous necropsy related queries. I feel very privileged to have worked with such an amazing institution. I would like to thank the members of the Sea Lion Cancer Consortium in particular Kathleen Colegrove, Karina Acevedo-Whitehouse, Matthew Breen and James Wellehan for their help with various aspects of this project.

I would also like to thank Jeff Graves for kindly allowing me to work in his lab along with Valentina Islas, Tanya Sneddon and Dave Forbes for their help and advice especially during the times when things didn't go quite right.

My gratitude goes to Karen Billington and Jeanie Finlayson for their time and patience in helping me with lab techniques that were new to me (Southern blot and immunohistochemistry). In addition I'd like to thank Mark Dagleish for both making me welcome in his lab and for his advice.

I would like to thank the members of the SHEILAS group; Joanne Kershaw, Silje-Kristin Jensen, Kelly Robinson and Johanna Baily for lightening the mood and helping me keep up my enthusiasm, but not least for making a day learning R fun. I would also like to pass my thanks to Johanna Baily in particular, for her patience in teaching me aspects of histology and immunohistochemistry and especially for that much needed chocolate bar following a day staring down the microscope.

This work was made possible by funding from NERC, SMRU Ltd and the John H Prescott Marine Mammal Rescue Assistance Grant Program and I would like to extend my gratitude to them all.

Finally I would like to thank my husband Andy for his support and above all patience!

Dedication

To my Grandparents and the Provans that were here before me

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Chapter 1

General Introduction

1.1 Cancer in humans and domestic animals

Over the last 100 years there have been enormous advances in the field of human medicine and health care. Conditions which previously resulted in premature mortality rarely now do so in the developed world. This change is driven by improvements in facilities for disease control, prevention and detection along with the development of vaccines and antibiotics enabling previously fatal conditions to be treated or managed (Tu, 2010, Hicks and Allen, 1999, Weatherall et al., 2006). Conversely to this, the incidence of morbidity and mortality due to neoplastic conditions in humans has increased through the twentieth century and recent predictions (in the UK) have identified, that this trend regarding certain cancers, will continue. The increase in number has been attributed to both life style and increasing age and size of the population (Tu, 2010, Mistry et al., 2011).

In domestic animals cancer is also frequently diagnosed (Kidd, 2008, Knottenbelt, 2003, Misdorp, 1996) and is noted to affect a variety of body tissues (Paoloni and Khanna, 2008). In companion animals, greater owner expectations regarding health care for their pets has encouraged further research into diagnosis and treatments of these conditions (Dobson, 2013, Stoewen, 2012, Villalobos and Kaplan, 2007). Although advances have trailed behind that of human cancer research, in recent years studies have identified significant similarities between certain cancers in humans and those found in animals (Airley, 2012, Paoloni and Khanna, 2008). Examples include comparable gene expression patterns in human and canine osteosarcoma and mutations identified in a tyrosine kinase growth factor receptor occurring in both human gastrointestinal cancer and in canine mast cell tumours (Paoloni and Khanna, 2008, Airley, 2012, Hirota et al., 1998, London et al., 1999, Mueller et al., 2007). Recognition of cases such as these has highlighted the importance of comparative oncology and what can be achieved by implementing a "one health" approach to medicine (Airley, 2012, McAloose and Newton, 2009, Munson and Moresco, 2007).

1.2 Cancer in wild animals

As in domestic animals cancer does occur in wild species, both in the captive and free-ranging situation (McAloose and Newton, 2009, Lombard and Witte, 1959). Neoplasms have been identified in a number of captive wild animal species belonging to various taxa during routine necropsy examinations at zoological institutes (Lombard and Witte, 1959, Ratcliffe, 1933, Effron et al., 1977). In some instances the cancer has been recognised to be a result of a management technique such as in the case of mammary carcinomas in zoo felids treated with the contraceptive melengestrol acetate (McAloose et al., 2007, Harrenstien et al., 1996). In free-ranging wild animals the identification of neoplasia is more challenging and relies on access to carcasses fresh enough to be adequately examined, therefore detection of cancer in wild animal species tends to be lower than in domestic animals (McAloose and Newton, 2009). In spite of these difficulties there are a few cases in the literature where certain forms of cancer are reported in a large number of individuals of a wild free-ranging population, these cancers and the species they affect are detailed below.

The endangered marsupial the Western barred bandicoot (*Perameles bougainville*) suffers from a neoplastic condition called papillomatosis and carcinomatosis (Woolford et al., 2007). The condition is associated with infection with a novel virus called bandicoot papillomatosis carcinomatosis virus type 1 (BPCV1) (Woolford et al., 2007). Affected animals have lesions varying in severity from wart like papillomas to squamous cell carcinoma affecting both skin and mucosal areas (Woolford et al., 2008). In addition to the identification of the disease in the Western barred bandicoot, a Southern brown bandicoot (*Isoodon obesulus*) belonging to the same Peramelidae marsupial family, was found to be suffering from similar lesions. PCR analysis subsequently identified a related virus designated bandicoot papillomatosis carcinomatosis virus type 2 (BPCV2) (Bennett et al., 2008). Genetic analysis of BPCV1 and BPCV2 indicated that they were novel, however they had genomic similarities with both papillomaviruses and polyomaviruses suggesting either a common viral ancestor or viral recombination (Bennett et al., 2008, Woolford et al., 2007).

Viruses have been associated with other wildlife cancers including herpesvirus associated fibropapillomatosis in various species of sea turtle (Quackenbush et al.,

1998). Fibropapillomatosis in sea turtles is characterised by multiple lesions affecting various regions of the body from the integument and eyes to internal organs (Brooks et al., 1994, Jacobson et al., 1991, Harshbarger, 1991). The consequence of these space-occupying lesions depends on their location, as growth progression of a lesion can severely compromise an animal's ability to function normally (Brooks et al., 1994, Herbst, 1994). The herpesvirus believed to be involved in the aetiology of the disease is related to alphaherpesviruses (Quackenbush et al., 1998, Greenblatt et al., 2005). Viral particles presumed to be herpesvirus were first identified from cutaneous lesions by histopathology and electron microscopy (Jacobson et al., 1991), the presence of herpesvirus DNA in affected tissues from four species of sea turtle was later identified by PCR (Quackenbush et al., 1998).

The Western barred bandicoot is not the only endangered marsupial to suffer from a high presence of cancer as carcinoma in a second endangered marsupial has received much attention in recent years. The Tasmanian devil (*Sarcophilus harrisii*) is a carnivorous marsupial found on the Island of Tasmania, that is affected by a metastatic tumour of neuroendocrine origin (Loh et al., 2006b, Loh et al., 2006a, Murchison, 2009). The disease termed Devil Facial Tumour Disease (DFTD) was first reported in the late 1990's and has now caused a 60% decrease in the population. If mortality from this disease continues at the current rate; the species is expected to become extinct in the wild within 20 years (Jones et al., 2007, McCallum, 2008).

DFTD is a transmissible allograft tumour where the tumour cells themselves act as an infectious agent, the only other cancer identified in the wild with this trait is canine transmissible venereal tumour (CTVT) (McCallum, 2008, Siddle et al., 2007, Murchison et al., 2012, Murchison, 2009). Spread of DFTD is believed to be via biting and possibly through cannibalism (Jones et al., 2007), it initially develops in the soft tissues of the face and is always fatal (Murchison, 2009). As the disease progresses the lesions affecting the facial tissues can become so severe that the animal is rendered unable to feed and can die of starvation. It additionally has the ability to metastasise and commonly spreads to lymph nodes, lung and spleen along with other organs also resulting in death if the animal has not already succumbed (Murchison, 2009, Loh et al., 2006a).

Cytogenetic analysis carried out on the DFTD tumour cells revealed large chromosome aberrations with one study identifying the apparent loss of five chromosomes and the gain of four abnormal chromosomes (Murchison, 2009). Clonality of the tumours was confirmed via microsatellite analysis, along with allele analysis at four major histocompatibility (MHC) loci (Murchison, 2009). Although steps have been made in identifying the origin of the cancer (Murchison et al., 2012), research has also suggested that loss of diversity of MHC may be a contributing factor (Siddle et al., 2007). Management strategies in order to save the species include establishing "insurance" populations of disease free animals and potentially the development of a vaccine, although the latter may be challenging due to the recent discovery of tumours cytogenetically different from the original strain (Pearse et al., 2012, Deakin et al., 2012).

1.3 Cancer in marine mammals

In marine mammals the range of species where neoplastic conditions have been reported is large, encompassing all the families within the marine mammal group. However in many cases neoplasia is an incidental finding and accounts of a particular condition are solitary, therefore gauging the true level of neoplasia in marine mammal species is difficult (Newman and Smith, 2006). Detection is hindered by the habitats they occupy which make surveillance logistically difficult (Gulland and Hall, 2007), as animals dying at sea may never be recovered. Additionally in the event of a carcass being salvaged it frequently is found to have undergone severe post mortem change, making a viable diagnostic necropsy impossible (Newman and Smith, 2006).

There are two species however where neoplasia is recognised in higher numbers; the California Sea Lion (*Zalophus californianus*) – the species of interest in this study and the Beluga whale (*Delphinapterus leucas*) (Newman and Smith, 2006). Necropsy examinations undertaken on stranded Beluga whales from the St Lawrence estuary in Canada have identified them as having a high prevalence of tumours (Martineau et al., 2002b). The tumours are found to mainly be gastrointestinal epithelial cell tumours and not haemopoietic tumours which are more commonly identified in cetaceans (Martineau et al., 2002b). The St Lawrence Estuary suffers from a high level of pollution and it is postulated that this is a major factor causing this phenomenon (De Guise et al., 1994,

Martineau et al., 2002b, Newman and Smith, 2006). Indeed elevated levels of organochlorines (OCs) and polycyclic aromatic hydrocarbons (PAHs) have been identified in tissues from St Lawrence Beluga whales in comparison to the levels found in Beluga whales from the Arctic (Newman and Smith, 2006, Metcalfe et al., 1999). The greater number of gastrointestinal tumours have been attributed to feeding on PAH contaminated fish (Martineau et al., 2002a), as the route of exposure to a contaminant can determine the type of tumour that develops. This was demonstrated in a number of experiments where mice were administered PAH by various routes resulting in different tumours (Culp et al., 1998). The involvement of contaminants in cancer affecting California sea lions has also been investigated and is discussed in section 1.5.4.

1.4 The California sea lion (*Zalophus californianus*)

1.4.1 The California sea lion

The California sea lion (*Zalophus californianus*) is a large carnivorous mammal of the suborder pinnipedia, which includes seals, fur seals and walruses. They are placed within the group Otariidae which like other pinnipeds arise from the extinct arctoid carnivores (Higdon et al., 2007). It is estimated that there are over 200,000 animals within the population that are found mainly along the west coast of America with a range from as far south as the Mexican Baja coast to as far north as British Columbia (Heath and Perrin, 2009) and as with other marine mammals they are protected under the Marine Mammal Protection Act (MMPA) (Moore et al., 2013).

California sea lions (CSL) feed on a variety of fish species and cephlopods but will also consume crustaceans if other food sources are unavailable (Heath and Perrin, 2009). They are particularly social animals and group together at haul out sites often exhibiting close contact (Heath and Perrin, 2009, Reidman, 1990a). A polygymous mating system is present and during the breeding and pupping season (May to July), males establish territories in order to mate with females within those areas (Gerber et al., 2010, Heath and Perrin, 2009). In addition CSL exhibit philopatry; returning to their birth place to breed (Miller, 2009). Male and female CSL reach sexual maturity at four to five years old, however they may not breed successfully until they are older (Heath and Perrin, 2009). Female CSL are monoestrus and ovulate approximately one month after

pupping. Gestation is 11 months which includes a three month period of embryonic diapause (delayed implantation) (Reidman, 1990b, Robeck et al., 2001). Delayed implantation is common to all pinnipeds however the length of time between conception and implantation varies between species (Robeck et al., 2001).

The life span of wild CSL has been reported as 15-24 years (Heath and Perrin, 2009). However in the absence of birth data precise aging of free ranging wild marine pinnipeds is difficult. In lieu of this standard body measurements and assessment of pelage are used as a guide to provide age ranges (Jeglinski et al., 2010, Wilson, 1974). In addition, measurement of dentine growth layers in teeth to predict the age of animals is also possible (Jeglinski et al., 2010, Mansfield and Fisher, 1960). Table 1.1 presents an outline of the morphometric classification of age ranges used to define CSL admitted to The Marine Mammal Center (TMMC), Sausalito, California. TMMC is a large marine mammal rehabilitation organisation that rescues between 600 and 800 animals a year; it additionally has a strong research focus, investigating a wide range of aspects concerning marine mammal health and disease. TMMC were major collaborators in this study.

Table 1.1: Guide to aging of CSL via morphology

Sex	Age class	Approx. Age (years)	Morphology
Female	Yearling*	1-2	Incisors and canines the same length
	No Juvenile Category	-	-
	Sub-adult	3-5	Canine larger
	Adult	5+	>150cm in body length
Male	Yearling*	1-2	Incisors and canines the same length
	Juvenile	2-4	Canine larger
	Sub-adult	4-8	Small sagittal crest present
	Adult	8+	Full sagittal crest present

*Using the assumption all CSL are born on 15th of June (Data courtesy of Lauren Rust, The Marine Mammal Center, personal communication)

1.4.2 Diseases of the California sea lion

Wild CSL suffer from a range of disorders. In an analysis of over 3000 animals stranded between 1991 and 2000 the most common conditions identified were malnutrition (32%), leptospirosis (27%), trauma (18%), domoic acid toxicity (9%) and cancer (3%) (Greig et al., 2005). Cases of malnutrition were highest during the El Niño years (1992, 1993 and 1998) when the food supply was depleted (Greig et al., 2005, Melin et al., 2010).

Leptospirosis in CSL results in interstitial nephritis which can progress to renal failure if left untreated (Cameron et al., 2008). The aetiological agent is *Leptospira interrogans* serovar Pomona, a spirochete bacteria with a postulated route of transmission via the urine (Cameron et al., 2008, Norman et al., 2008). Outbreaks of leptospirosis occur yearly between the months of June and December, however every three to five years epizootics of a larger scale occur which result in significant mortality (Cameron et al., 2008, Lloyd-Smith et al., 2007).

Incidents of trauma are frequent and include anthropogenic causes such as entanglement in fishing line, injuries due to propellers and gunshot wounds along with more natural causes such as shark bites (Greig et al., 2005, Moore et al., 2013). Domoic acid toxicity was first diagnosed in 1998, domoic acid is a neurotoxin produced by the marine diatom *Pseudo-nitzschia*, ingestion of toxin results in seizures that can progress to coma and death (Goldstein et al., 2008). Domoic acid toxin is similar in structure to the neurotransmitter glutamic acid; the structural similarity enables it to bind glutamate receptors in the brain causing excitement. Since its identification cases have increased with increases in blooms of *Pseudo-nitzschia* (Goldstein et al., 2008, Mos, 2001).

Other documented conditions include infection with a variety of agents (Greig et al., 2005, Thornton et al., 1998). Bacterial infections secondary to trauma or parasitic infection can result in serious conditions such as pneumonia and peritonitis (Greig et al., 2005). Parasitic infections with nematodes including parafiliroides and anisakid species are reported to contribute towards micro-abscesses in the lungs and gastric ulcers respectively (Greig et al., 2005, Kelly et al., 2005). Diseases due to a number of viruses are reported, a common example is a Calicivirus called San Miguel Sea Lion Virus

which can cause a range of disorders from ulcerations of the skin and mucosal regions to encephalitis (Li et al., 2011). Viruses have also been isolated from neoplastic conditions and these are summarised in Table 1.2, however further work is required to confirm causation. Neoplasms in individual CSL are sporadically reported throughout the literature affecting both wild and captive animals; examples of these are also detailed in Table 1.2. The table is by no means exhaustive as reports of over 10 types of cancer affecting greater than 20 different tissues have been made as reviewed by Newman and Smith, 2006 (Newman and Smith, 2006). The most predominant cancer identified is urogenital carcinoma (UGC) (Newman and Smith, 2006) and is the subject of this study.

The reports of various cancers in captive CSL appear to be more frequent in older animals (Table 1.2); this is consistent with what is reported in companion animals and other captive wild animals possibly due to their longer life expectancy (Lohmann, 2007, Martineau et al., 2002b, Courtenay and Santow, 1989, Lombard and Witte, 1959, Paoloni and Khanna, 2008). The aging process is associated with a higher occurrence of cancer due to the increased time allowing the accumulation of DNA mutations (Dunn, 2012). In many cases the literature does not state whether the animal was captive bred or originated from the wild. In the case of UGC there are many reports of the disease in wild animals as will be discussed further, in addition to this captive bred animals are also reported to suffer from the condition alongside captive wild-born animals (Dr Michelle Davis, SeaWorld Orlando, personal communication).

Table 1.2: A selection of neoplasms reported in CSL in the wild and in captivity

Type of cancer	Captive or wild?	Virus isolated?	Comment	Reference
Fibropapilloma on tongue and T Cell intestinal lymphoma	Wild	Polyomavirus designated California sea lion polyomavirus 1.	Lymphoma induction via the virus was considered unlikely	(Colegrove et al., 2010)
B cell lymphoblastic lymphoma	Captive (24 yrs old)	Otarine herpes virus - 3	More studies required to assess the potential link	(Venn-Watson et al., 2012)
Urogenital carcinoma*	Wild and Captive	Otarine herpes virus - 1	On-going studies	(Gulland et al., 1996, King et al., 2002, Lipscomb et al., 2000, Buckles et al., 2006)
Metastatic adenocarcinoma affecting lymph nodes, lung, liver, kidney and spleen	Wild	Not reported	Primary site of cancer not identified, but suggestion of genital tract origin	(Brown et al., 1980)
Metastatic squamous cell carcinoma affecting the lymph nodes, lungs, liver, kidney and ovary	Wild	Not reported	Primary site of cancer not identified, but suggestion of genital tract origin	(Joseph et al., 1986)
Cutaneous squamous cell carcinoma	Captive (~18-20 yrs old)	Not reported	Locally invasive, no evidence of metastasis	(Anderson et al., 1990)

*Multiple cases of this type of neoplasia

Table 1.2 cont.

Type of Cancer	Captive or wild**?**	Virus isolated?	Comment	Reference
Multicentric neurofibromatosis	Captive (~31 yrs old)	Not reported		(Rush et al., 2012)
Gingival squamous cell carcinoma	Captive (~30 yrs old)	Not reported	Locally invasive, no evidence of metastasis	(Bossart, 1990)
Metastatic hepatic carcinoma with spread to spleen	Wild	Not reported	Hepatic origin	(Acevedo-Whitehouse et al., 1999)
Mammary carcinoma with metastasis to regional lymph nodes	Captive (~28 yrs old)	Not reported	Mammary origin	(Matsuda et al., 2003)

1.5 Disseminated carcinoma of urogenital origin in the California sea lion

CSL appear to have a particularly high occurrence of UGC within their population. This was highlighted initially by a study of the occurrence of neoplasia in this species in animals presented to TMMC between 1979 and 1994 (Gulland et al., 1996). Of the 370 sub-adults and adults examined in the 15 year period via necropsy and histology, 18% were found with the presence of metastatic carcinoma. It was additionally stated that during the earlier four years of the study, the true number of cases may actually have been higher as the necropsies preformed during this time had been less meticulous (Gulland et al., 1996). Since that study monitoring of the level of UGC has continued and in the 15 years between 1998 and 2012 has shown an overall increase in prevalence to 26%. During this time 931 dead adult CSL were examined of which 205 were diagnosed with UGC (Dr Frances Gulland/TMMC, personal communication).

Due to the nature of marine mammal habitats it is not possible to establish the true prevalence of UGC in the wild population as animals that die at sea may never be recovered. It should be remembered that the prevalence stated here is calculated from animals admitted to TMMC only, however the findings of this study indicate that UGC is an important cause of morbidity and mortality in this species (Gulland et al., 1996).

1.5.1 Signalment

The disease affects sub-adults and adult animals of both sexes of a mean age of approximately eight years old (Gulland et al., 1996, Buckles et al., 2006). In the wild CSL can live into their 20's therefore this is not a cancer typical of old age (Gulland et al., 1996). In this way the condition mirrors the situation in human cervical cancer, where cases tend to occur in adult but not necessarily aged women (Hemminki et al., 2001, Gustafsson et al., 1997).

1.5.2 Clinical presentation

Affected animals can present with a variety of clinical symptoms including cachexia, hind flipper paresis, ascities, hind flipper and perineal oedema (Figure 1.1), in addition to these in severe cases rectal prolapse may occur (Gulland et al., 1996).

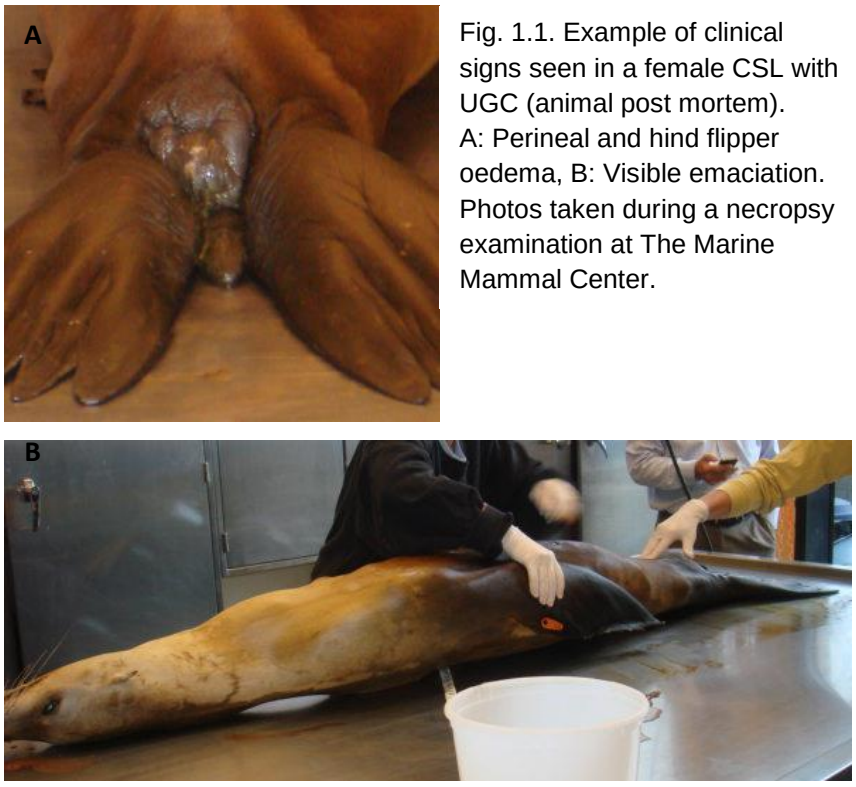


Fig. 1.1. Example of clinical signs seen in a female CSL with UGC (animal post mortem). A: Perineal and hind flipper oedema, B: Visible emaciation. Photos taken during a necropsy examination at The Marine Mammal Center.

1.5.3 Pathology

1.5.3.1 Gross pathology

On gross pathological examination a range of lesions may be identified affecting both the genital tract and the rest of the body. Abnormalities in the genital tract may be present (Figure 1.2), however in some cases lesions may not be obvious and neoplasia may be diagnosed incidentally by histopathology of genital tissue (Gulland et al., 1996). In advanced cases metastasis is common and lesions are noted in a number of sites including the abdominal and pelvic lymph nodes along with more distant sites such as the liver (Figure 1.2), lungs and spleen. The renal system is frequently affected, with metastatic lesions present in the kidneys along with bladder distension and in some instances hydroureter and hydronephrosis (Gulland et al., 1996).

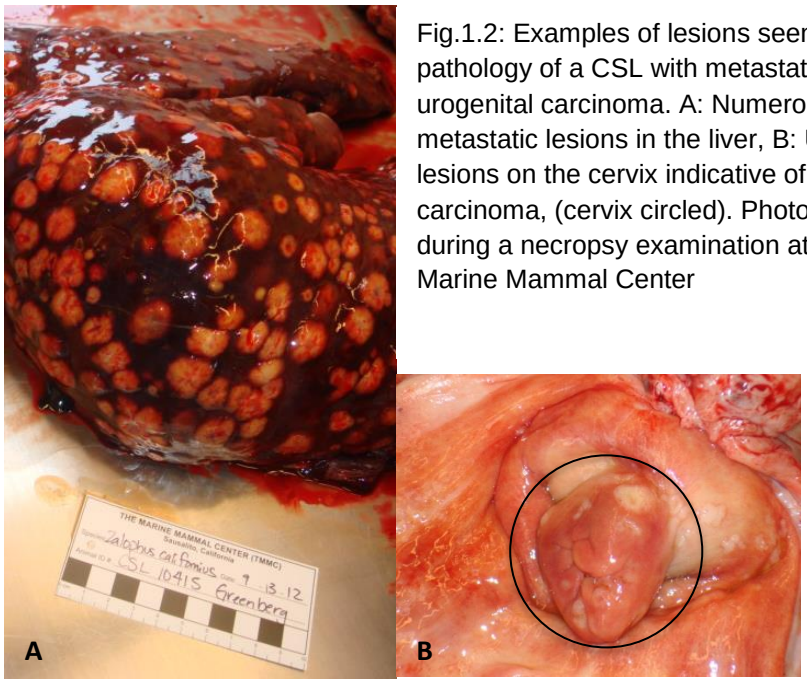


Fig.1.2: Examples of lesions seen on gross pathology of a CSL with metastatic urogenital carcinoma. A: Numerous metastatic lesions in the liver, B: Ulcerative lesions on the cervix indicative of urogenital carcinoma, (cervix circled). Photos taken during a necropsy examination at The Marine Mammal Center

1.5.3.2 Histopathology

Initial work suggested that the origin of the tumour was the urinary tract, based on histological findings of the presence of presumed transitional cells (Gulland et al., 1996), however later work identified genital epithelial lesions which corresponded to lesions known as intraepithelial neoplasia (IEN) (Lipscomb et al., 2000). IEN lesions also occur in humans and are frequently associated with cervical cancer where they are classified according to the cervical intraepithelial neoplasia (CIN) grading system and range from CIN I (mild) to CIN III (carcinoma *in situ*) (Crum, 2005, Herbert et al., 2007, Buckley et al., 1982). CIN III is considered to be a predictor of probable invasive carcinoma (McCredie et al., 2008, Crum, 2005). The grades describe the level of cellular dysplasia when changes are restricted to the epithelial layer (i.e. they are "intraepithelial" lesions) once the basement membrane is breached however, invasive carcinoma ensues (Liotta, 1984, Stewart and McNicol, 1992). The identification of IEN in genital tissue points towards a genital origin of the disease alone rather than a urinary tract origin as was previously suggested (Lipscomb et al., 2000, Lipscomb et al., 2010).

In the CSL the classification of histopathological lesions uses the terminology; low-grade intraepithelial (LGIL) lesions, which encompasses CIN I and high-grade intraepithelial (HGIL) lesions encompassing CIN II and III as opposed to the CIN grading system. LGIL and HGIL are used in preference as lesions identified are not restricted to the cervix as they have been reported in the vagina, penis and prepuce (Dr Kathleen Colegrove, personal communication (Colegrove et al., 2009)). In addition to this LGIL and HGIL have been identified in urethral tissue, suggesting that the point of origin of the disease still requires clarification (Colegrove et al., 2009, Lipscomb et al., 2010). The majority of carcinomas identified in CSL are squamous cell carcinomas however adenocarcinomas have also been noted (Colegrove et al., 2009, Gulland et al., 1996). Examples of different histological findings are shown in Figure 1.3.

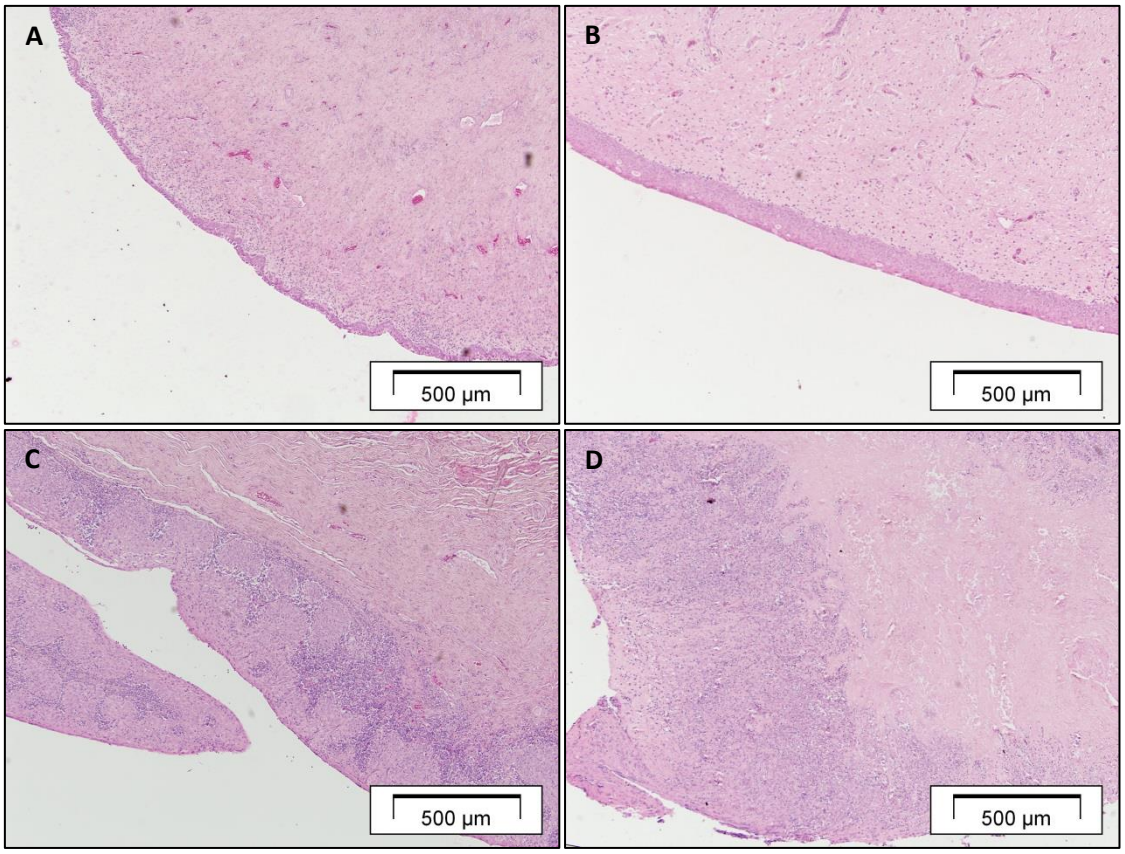


Fig. 1.3. Haematoxylin and eosin stained sections illustrating various pathologies in samples taken from CSL from normal cervix (A) to infiltrative carcinoma in (D). A] Normal cervix B] Low grade intraepithelial neoplasia showing mild dysplasia in the epithelial layer ; C] High grade intraepithelial neoplasia showing dysplasia affecting the full thickness of the epithelial layer; D] Infiltrative carcinoma in showing breach of the basement membrane. (Sections courtesy of Dr Kathleen Colegrove, Veterinary Diagnostic Laboratory, University of Illinois, USA and prepared by Ms Jeanie Finalyson, The Moredun Research Institute, Edinburgh, UK).

1.5.4 The story so far…

Cancer is a multifactorial disease making the identification of the aetiology of a particular neoplasm challenging (Stanhope et al., 1964, Bunz, 2008). In some cases the cause has been identified as with transmissible allograft cancer in DFTD and CTVT (McCallum, 2008, Murchison, 2009, Murchison et al., 2012, Siddle et al., 2007). In other instances tumours are associated with viruses such as BPCV1 and herpes-associated fibropapillomatosis mentioned earlier, however the presence of viral infection does not necessarily result in cancer development suggesting the role of other factors are at play (Morris et al., 1995). In other cases the relationship with a causal

agent remains only strongly associative as in the Beluga whales exposed to pollution in the St Lawrence estuary (Newman and Smith, 2006, Martineau et al., 2002b, De Guise et al., 1994).

In order to further investigate UGC in the CSL the Sea Lion Cancer Consortium (SLiCC) was established in 2010 (http://www.smru.st-andrews.ac.uk/slicc/ accessed on 19/02/14). To date, the studies carried out have covered four main areas as potential factors involved in the aetiology of this disease; exposure to contaminants, hormone receptor expression, infectious agents and genetic factors.

1.5.4.1 Exposure to contaminants:

The link between certain contaminants and cancer has been known since the 18th century when Sir Percival Pott in 1775 identified that a high number of chimney sweeps were suffering from scrotal cancer and concluded that it was associated with soot collecting in the rugae of the scrotum reviewed by Brown and Thornton, 1957 (Brown and Thornton, 1957). In modern times one of the best known links regarding contaminants and neoplasia is that of lung cancer and cigarette smoke (Hecht, 1999, Stanhope et al., 1964, Doll and Hill, 1954). Cigarette smoke has additionally been implicated in other cancers including cancer of the colon, breast, cervix and bladder (Botteri et al., 2008, Gaudet et al., 2013, Brennan et al., 2000, Trimble et al., 2005).

PAHs and OCs have been mentioned earlier with regards to the high number of cancers identified in the St Lawrence Beluga whales (Metcalfe et al., 1999, Newman and Smith, 2006). The PAHs have a direct genotoxic effect by the formation of DNA adducts, where the compound becomes covalently bonded to DNA resulting in structural deformities thus predisposing the cell to altered gene expression (Weinstein, 1988, Farmer, 2004). Additionally increased levels of OCs such as polychlorinated biphenyls (PCBs) and dichloro-diphenyl-trichoroethane (DDT) have been associated with genetic mutations and tumour promoter activity (Howsam et al., 2004, Porta et al., 1999, Scribner and Mottet, 1981). Environmental studies have identified OC and PAH pollutants in coastal waters around California (Oros et al., 2007, Zeng and Venkatesan, 1999, Schiff et al., 2000) and previous work has found the presence of these compounds in tissues from CSL (Le Boeuf and Bonnell, 1971, Le Boeuf et al., 2002b, Colegrove,

2008). In light of this the level of OCs in the blubber of CSL diagnosed with UGC was compared to the level in blubber from non-cancer animals with an increased level in cancer animals being identified (Ylitalo et al., 2005). This suggested an association between the level of contaminant and the presence of cancer, however confounding factors such as the effect of changing body condition and blubber dynamics need to be understood before this relationship is verified (Ylitalo et al., 2005). PAH related pathology in the form of PAH-adducts have been identified in the liver of CSL however as yet a link with neoplasia has not been established (Colegrove, 2008).

1.5.4.2 Reproductive hormone receptor expression:

Oestrogen and progesterone receptors have previously been identified as prognostic markers in assessing breast cancer in humans (Murphy and Watson, 2002, Vollenweider-Zerargui et al., 1986). Loss of expression of estrogen receptor alpha (ER-α) and/or progesterone receptor (PR) is seen to indicate poor prognosis (Vollenweider-Zerargui et al., 1986). A recent study however has questioned the merit of measuring progesterone receptor expression in assessing prognosis in ER positive breast cancer, as although PR expression holds prognostic value, the expression of other genes examined in the study were identified as being more strongly associated with prognosis (Hefti et al., 2013). The findings suggested that other genetic markers may be of more value in assessing ERα positive breast tumours. In the case of ERα negative tumours PR expression wasn't found to be associated with prognosis (Hefti et al., 2013).

Studies in animals have identified similar associations. Increased ER-α and PR expression were found to be correlated with a better prognosis in canine mammary tumours (Nieto et al., 2000, Mariotti et al., 2013). In rabbits with uterine adenocarcinoma however the situation was different and expression of ERα and PR was not seen to reflect prognosis (Asakawa et al., 2008). Rabbits have a high occurrence of metastatic uterine adenocarcinoma with the disease frequently affecting animals of approximately five years old (Asakawa et al., 2008, Baba and von Haam, 1972, Greene and Saxton, 1938). Two histological types of adenocarcinoma (papillary and tubular/solid tumours) have been identified exhibiting different expression patterns of ERα and PR. It is postulated that the varying hormone receptor expression plays a role in the development of the different types of tumours identified (Asakawa et al., 2008).

In addition there appears to be a breed predisposition to the disease (Baba and von Haam, 1972). Hormone receptor expression in the CSL has been investigated in CSL with UGC using immunohistochemistry (Colegrove et al., 2009). In a recent study no difference in PR expression was identified between tumour affected tissues and normal tissues, however oestrogen receptors were found to be reduced in affected tissue (Colegrove et al., 2009). This reduction coincided with increased Ki67 (cell proliferation) index and p53 expression. The protein p53 is the product of expression of the tumour suppressor gene *P53*, the increased labelling identified suggests that it may be involved in cancer pathogenesis. (Colegrove et al., 2009).

1.5.4.3 Infectious agents:

To date two infectious agents have been linked to UGC in the CSL; Otarine herpes virus 1 (OtHV-1), a gamma herpesvirus (Lipscomb et al., 2000, King et al., 2002, Buckles et al., 2006) and beta (β) haemolytic *Streptococcus*. The association of OtHV-1 and UGC is discussed in greater detail in Chapter 6. Studies into cervical bacterial flora in humans have identified an association with abnormal flora and the presence of CIN (Guijon et al., 1992). Similarly investigations in CSL have identified a β-haemolytic *Streptococcus* as being significantly associated with the presence of UGC in females only (Johnson et al., 2006). However, the true nature of this association has yet to be determined (Johnson et al., 2006).

1.5.4.4 Genetic factors:

Cancer is essentially a genetic disease occurring as a result of loss of control of the cell cycle and research into the genetic basis of various cancers is the main focus of many studies today (Weinstein and Case, 2008, Bunz, 2008). To rule out large scale chromosome aberrations such as those present in DFTD and CTVT, karyotyping of the CSL genome was carried out to compare the karyotype from cells from normal tissue with those affected with cancer. No abnormalities have been identified in karyotype number, however intra-chromosomal copy number aberrations were noted and this is a continued area of investigation (Breen, 2011). Loss of diversity at the MHC loci has been identified as important in cancer in the Tasmanian devil (Siddle et al., 2007), likewise an association has been identified in the CSL, where a specific MHC class II

locus (Zaca-DRB.A) is seen as important with regards to the presence of UGC. The DRB family of genes in the CSL consists of eight loci designated Zaca-DRB.A to Zaca-DRB.H (Bowen et al., 2004, Bowen et al., 2005) and varying combinations of these have been identified. However the presence of the Zaca-DRB.A locus in any combination was found to be significantly associated with the presence of UGC (Bowen et al., 2005). A final genetic association has been identified in the CSL indicating a potential effect of inbreeding with susceptibility to cancer (Acevedo-Whitehouse et al., 2003). The study incorporated microsatellites as genetic markers and resulted in the identification of two microsatellites of particular interest called M11a and Pv11 (Acevedo-Whitehouse, unpublished). Subsequent work involving the Pv11 microsatellite led to the identification of a potential gene of interest in UGC called heparanase 2 (HPSE2) (Acevedo-Whitehouse and Hammond, unpublished). The relevance of these microsatellite markers along with the potential importance of the HPSE2 gene in UGC will be discussed further in subsequent chapters.

1.6 Summary

The CSL shares much of its coastal habitat and diet with humans and can be considered as a sentinel species for the health of the marine environment (Bossart, 2006). UGC in CSL is an important cause of morbidity and mortality with environmental, genetic and infectious factors potentially all playing a role in its development. Determining the aetiology of the condition is clearly important for future management and conservation plans for this protected species.

1.7 Project aims and thesis structure

Due to the complexities of neoplasia there are many possible avenues of investigation of this disease, however it is not possible to address them all, instead I have focussed on two areas of investigation; particular genetic factors (Chapters 2-5) and herpesvirus infection (Chapter 6). The overall objectives of each chapter are as follows: Chapter 2 - To ascertain the true nature of the relationship between the genetic markers previously identified as important (Acevedo-Whitehouse, unpublished) and the occurrence of UGC using a case-control study design; Chapter 3 – To confirm the location of one of these markers within the CSL genome; Chapter 4 – To investigate the possibility of a

genetic mutation, utilizing a single genetic marker; Chapter 5 - To investigate the activity of the HPSE2 gene in the CSL; Chapter 6 - To determine herpesvirus prevalence in the study animals, again through a case control study. The final chapter (Chapter 7) collates the information gained throughout the study in order to draw conclusions and determine appropriate areas of future investigation.

The aims detailed above for each chapter assist in offering support for a general hypothesis for the thesis:

" Urogenital carcinoma in the California sea lion has a multi-factorial aetiology including a genetic and infectious basis"

In order to support this hypothesis the following study questions will be considered:

1. Is there an association between the genotype of certain genetic markers and the presence of UGC in the CSL?
2. If an association is identified with a genetic marker and the presence of UGC does it indicate a gene of interest?
3. Does genetic instability occur in CSL with UGC?
4. Is the presence of herpesvirus associated with the occurrence of UGC?

Chapter 2

Microsatellite genotype as a predictor for the presence of urogenital carcinoma in the California sea lion (*Zalophus californianus*).

2.1 Introduction

The investigation of the occurrence and causes of disease in free ranging wild populations of animals presents logistical difficulties. Identifying related animals in order to study the effects of inbreeding or to identify familial traits can be particularly challenging. Animals may be found in environments that are difficult to access, have a nocturnal existence or be particularly elusive (Witmer, 2005). The utilization of genetic microsatellite markers to identify related individuals has made keeping track of the dynamics of a population possible (Webster and Reichart, 2005, Jarne and Lagoda, 1996). Microsatellites are highly polymorphic repeat units of nucleotides of up to six base pairs (bp) and are reviewed by Ellegren, 2004 and Selkoe and Toonen, 2006. The repeats can occur between five to forty times at any one site in the genome and thousands of times at different sites throughout the genome (Ellegren, 2004, Selkoe and Toonen, 2006). It is estimated that microsatellites account for 3% of the human genome (Ellegren, 2004, Lander et al., 2001).

Polymorphism of microsatellite alleles are commonly due to variations in the length of the allele caused by variations in the number of repeat units, rather than as a result of variations in the actual repeated sequence (Ellegren, 2004). Their presumed neutrality, along with their polymorphic existence have resulted in their frequent use as markers in population genetic studies involving the investigation of population structure, paternity and gene flow in a number of species ranging from Zebra finches (*Taeniopygia guttata*) and Black-faced Lion tamarins (*Leontopithecus caissara*) to Blacktip Reef sharks (*Carcharhinus melanopterus*) and Caribbean star corals *(Montastraea faveolata*) (Davies et al., 2013, Vignaud et al., 2013, Martins and Galetti, 2011, Webster and Reichart, 2005, Dawson et al., 2013). The field of marine mammal research poses additional logistical difficulties due to the aquatic environment of the animals investigated, however obtaining DNA is possible and it has been extracted from various

sources including skin and blood (Bean et al., 2004, Yu et al., 2011, Torres-Florez et al., 2012). Samples such as these however require direct access to the animal which is not always possible, so additional non-invasive sampling methods have been employed such as extracting DNA from collected faeces or carcasses (Reed et al., 1997, Bean et al., 2004, Kretzmann et al., 2006). More recently studies into the merits of environmental DNA sampling using sea water have been investigated (Foote et al., 2012). These various methods of obtaining DNA have allowed population genetic studies using microsatellite markers on a variety of marine mammals, enabling a greater understanding of their populations (Buchanan et al., 1998, Graves et al., 2009, Bean et al., 2004, Torres-Florez et al., 2012).

Microsatellites are found in both coding and non-coding regions of DNA, however the majority are found in non-coding DNA such as intergenic sequences or introns (Ellegren, 2004) which are removed by RNA splicing prior to translation (Faustino and Cooper, 2003, Jaillon et al., 2008). In spite of this, polymorphisms within these microsatellites have increasingly been found to be important in genetic function (Li et al., 2004, Zhang et al., 2009). An example of this is the microsatellite found within the Epidermal Growth Factor Receptor gene (*EGFR*), where polymorphisms of the CA repeat in intron one is associated with differing clinical outcomes in non-small cell lung cancer (Shitara et al., 2012) and oesophageal cancer patients (Vashist et al., 2013). Additionally the presence of shorter CA repeats at this locus was identified as more common in osteosarcoma patients (Kersting et al., 2008). Similarly longer CA repeats in intron five of the oestrogen receptor gene *ESR2* have been identified as a risk factor in breast cancer in Nigerian women (Zheng et al., 2012). This challenges the argument of microsatellite neutrality (Li et al., 2002, Kashi and King, 2006).

Further to this microsatellites have been useful in detecting significant genetic associations to common diseases in humans and domestic animals (Gulcher, 2012). One such example is that of type 2 diabetes in humans where, along with obesity and life style, genetic factors have been highlighted as additional risk factors. Individuals with a history of the disease in their family have been identified as being at greater risk (Reynisdottir et al., 2003). Positional cloning through a genome-wide linkage study of Icelandic families led to the identification of the Transcription Factor 7-Like 2 gene

(*TCF7L2)*, variants of which are seen to be associated with the risk of developing type 2 diabetes (Reynisdottir et al., 2003, Grant et al., 2006). Associations have also been identified in neoplastic conditions. Certain alleles of the ZuBeCa3 microsatellite have been found to be associated with the presence of mammary tumours in various breeds of dog. The candidate gene of interest in this case is believed to be the Breast cancer-associated gene 1 (*BRCA1*) located in close proximity on chromosome 9 in domestic dogs. However, the relationship is yet to be confirmed (Bhattacharya et al., 2007).

The consequences of inbreeding in animal populations have also been assessed using microsatellites. Increased homozygosity of microsatellite alleles in inbred animals have been found to be associated with decreased fitness traits including reduced sperm quality, increased parasite load and presence of skeletal deformities (Rijks et al., 2008, Gage et al., 2006, Fitzpatrick and Evans, 2009, Lacy and Horner, 1996). Inbreeding depression (reduced fitness in a population) has important implications, as the tendency towards homozygosity due to mating of related individuals has the potential to reveal deleterious alleles along with the loss of heterozygous advantage (Lacy and Horner, 1996, Hansson and Westerberg, 2002, Charlesworth and Willis, 2009). However, with regards to studying inbreeding, the relationship between measures of microsatellite heterozygosity and fitness known as heterozygosity-fitness correlations are only found to be fulfilled under specific circumstances. For instance when populations are small or when mating systems include behaviours such as polygyny (Balloux et al., 2004, Fitzpatrick and Evans, 2009).

In the CSL microsatellite markers have been used to evaluate variation in the susceptibility to a number of diseases (Acevedo-Whitehouse et al., 2003) leading to the conclusion that morbidity in the species may not be a random event. The study incorporated 11 polymorphic microsatellite markers, which enabled the measurement of "internal relatedness" (Amos et al., 2001, Balloux et al., 2004) of 371 animals, 13 of which had a diagnosis of carcinoma of unspecified type. The animals had been admitted to the Marine Mammal Center in Sausalito, California, due to stranding. Internal relatedness (IR) is a measure of heterozygosity and can reveal inbreeding of an individual. The equation used $IR=2H-\Sigma fi / 2N-\Sigma fi$, where H is the number of loci that the individual is homozygote at and N is the number of loci genotyped, includes every

allele genotyped in an individual as well as in the study population, as Σ*fi* is the sum of the population frequencies of all the (*i*) alleles (Amos et al., 2001, Balloux et al., 2004). Therefore the IR calculation takes into account rare alleles in the population studied (Balloux et al., 2004, Amos et al., 2001). If the result of the calculation gives a positive number inbreeding is indicated, whereas a negative value suggests outbreeding and a zero result indicates that the parents were unrelated (Valimaki et al., 2007, Amos et al., 2001, Balloux et al., 2004).

The results of the study by Acevedo-Whitehouse et al., (2003) suggested that inbreeding may play a part in increased susceptibility to disease. An independent study confirmed that the CSL dataset published by Acevedo-Whitehouse and collaborators (2003) did indeed contain inbred individuals (Balloux et al., 2004), a phenomenon most likely explained by the species' strong polygyny and philopatry (Gerber et al., 2010, Miller, 2009, Young and Gerber, 2008, Heath and Perrin, 2009). Interestingly, the condition most highly associated with the internal relatedness measure was that of carcinoma. However the role of "in-breeding" per se is uncertain as further statistical analysis by Acevedo-Whitehouse (unpublished) found that the strength of the measure was driven by particular microsatellites in the animals with neoplasia, namely Pv11 (Goodman, 1997) and M11a (Hoelzel et al., 2001).

Therefore the aim of the present study was to (i) investigate the relationship between the Pv11 and M11a loci with the occurrence of UGC in a new sample population. The new sample set only consisted of adult female animals in order to remove the confounding factors of sex and age. The study also incorporated a third microsatellite, Hg8.10 (Allen et al., 1995) not previously associated with cancer, that was used as a control.

In addition to the dataset generated by this study, a second separate dataset was provided for analysis, the second dataset was genotyped by Dr Acevedo-Whitehouse and consisted of 270 adult and sub-adult animals of both sexes, 66 of which were suffering from UGC specifically.

2.2 Materials and Methods

2.2.1 Microsatellite genotyping

Sample collection:

Genotyping was undertaken on DNA extracted from skin samples. Samples were obtained by staff at The Marine Mammal Center (Sausalito, California, USA) during necropsy examinations of 113 female adult CSLs (Figure 2.1). The animals were admitted between October 2004 and December 2010. Adult status was assessed by body length, with animals >150cm considered as adults. Only adult female animals were included in the study to remove the confounding factors of sex and age. This is in comparison to the dataset compiled by Dr Acevedo-Whitehouse which consisted of adults and sub-adults of both sexes.

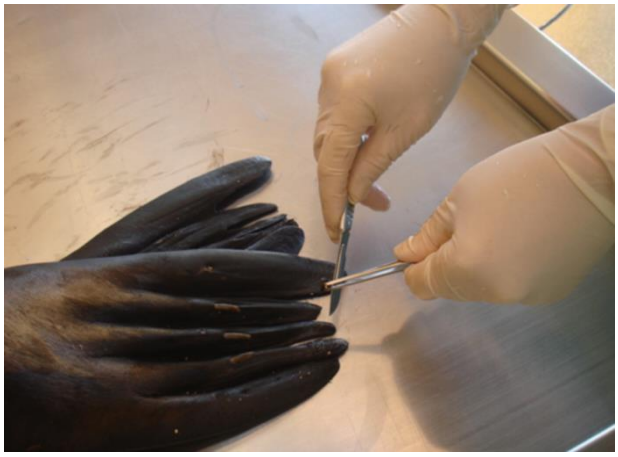


Fig 2.1: Obtaining a skin sample for DNA analysis during a necropsy examination at the Marine Mammal Center. Samples taken from the hind flipper.

The gross necropsy and histology reports were reviewed for all 113 animals allowing classification according to cause of death. UGC was diagnosed in 43 of the animals sampled and 70 were considered as control animals; having died or being euthanized due to a condition other than typical UGC. Cause of death in control animals is detailed in Figure 2.2 and Appendix A .

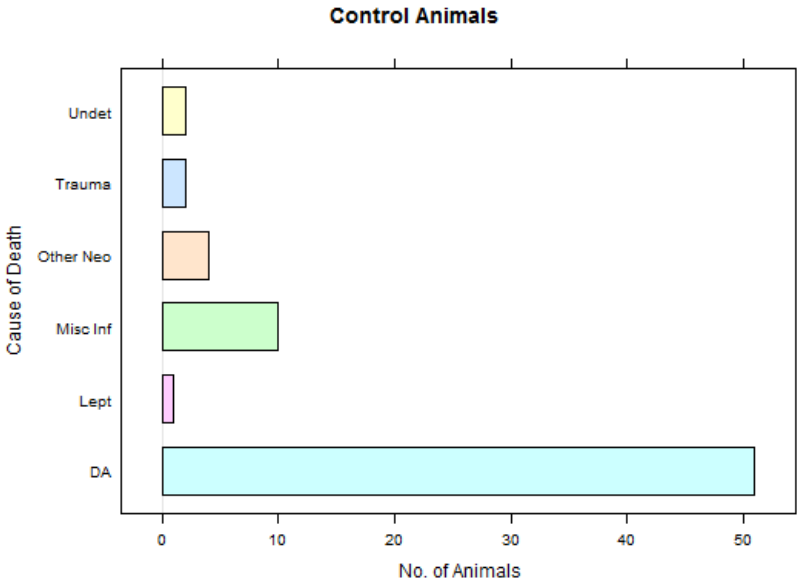


Fig. 2.2 Cause of death of control animals based on predominant necropsy finding recorded. (Undet: Undetermined, Other Neo: Neoplasia other than UGC identified; Misc Inf: Miscellaneous Infection; Lept: Leptospirosis; DA: Domoic acid). Undetermined indicates cases where a cause of death was not determined, however urogenital carcinoma (UGC) was not identified.

Information regarding which sample came from the UGC positive animals and which came from the control animals was not accessed until the analysis stage of these experiments was reached, to ensure unbiased data analysis.

The skin samples were stored in ethanol and sent to the Sea Mammal Research Unit (St Andrews, Fife) and on arrival they were stored at -20°C.

DNA extraction, purification and preparation:

DNA was extracted from the skin samples using the Puregene DNA isolation method detailed below in batches of 10-12 samples.

A small section (approximately 3 mm×3 mm) was cut from the skin samples and further sectioned with a sterile razor. The skin pieces were added to 600 µl of chilled cell lysis solution (0.1 M EDTA (VWR International Ltd, Poole, UK), 0.2 M Tris (VWR International Ltd) pH 8.5, 1% SDS (VWR International Ltd /BDH) in a 1.5 ml Eppendorf, 6 µl of Proteinase K (20 mg/ml) (Bioline Reagents Ltd, London, UK) was then added to the samples and they were incubated at 55°C with mixing overnight. If the samples had not digested thoroughly following overnight incubation they were

homogenised with a micropestle and a further 6 µl of Protinase K (20mg/ml) (Bioline Reagents Ltd, London, UK) was added followed by further incubation at 55°C (with mixing) for 60 min. Once digestion had taken place, 3 µl of RNase A (10 mg/ml) (Sigma-Aldrich Ltd, Gillingham, UK) was added and the samples were mixed by inverting them approximately 20 times. They were then incubated at 37°C for 60 min followed by cooling to room temperature and 200 µl of 5 M Potassium acetate (VWR International Ltd, Poole, UK) was added. The samples were mixed by vortexing at top speed for 20 s prior to being centrifuged at 17,000 × *g* for 3 min. The supernatant was decanted via pipette into a new 1.5 ml Eppendorf containing 600 µl of 100% isopropanol and the tubes containing the sediment were discarded. The supernatant and isopropanol were mixed by inverting gently until clumps of DNA could be seen. The DNA was pelleted by centrifuging at 17,000 × *g* for 1 min and the supernatant discarded. The pellet was washed by adding 600 µl of 70% ethanol, the sample was centrifuged at 17,000 × *g* for a further minute and the supernatant discarded. To ensure complete removal of all the ethanol the samples were dried in an incubator at 37°C. Re-suspension of the DNA was carried out by adding 100 µl of MilliQ water and incubating the samples overnight at room temperature.

Following re-suspension of the pellet, the quantity of DNA in the extraction samples was measured with a Nanodrop spectrophotometer (ND-1000, Thermo Fisher Scientific, Wilmington, USA) and working stocks were made by diluting the samples to 10 ng/µl by the addition of MilliQ water. The working stocks and the undiluted stocks were stored at -20°C.

Amplification of microsatellite markers:

Amplification of the three microsatellite markers; Pv11, M11a and the control microsatellite Hg8.10, was undertaken via a multiplex polymerase chain reaction (PCR). The protocol was adapted for the three microsatellites from one used previously to examine a number of microsatellite markers (including the three in the present study) in other marine mammal species by Dr Valentina Islas (University of St Andrews) following the Qiagen multiplex PCR kit protocol.

For the PCR, 10 µl reaction mixtures were prepared consisting of 20 ng DNA, 5 µl 2× Multiplex Master Mix (Qiagen, Crawley, UK) and 3 µl of primer mix. The primer mix was prepared by combining 6 pmol of forward primer and 6 pmol of reverse primer of the three microsatellites along with 3.3 pmol of Pv11 forward primer with a D3 fluorescent tag (green), 2.1 pmol of M11a forward primer with a D4 fluorescent tag (blue) and 3 pmol of Hg8.10 forward primer with a D2 fluorescent tag (black) and 2.1 µl RNase-free water (Qiagen, Crawley, UK). Primers were obtained from Invitrogen (Paisley, UK) and fluorescent tags from Sigma-Aldrich Ltd, (Gillingham, UK). Primer sequences are detailed in Table 2.1. Amplification of the microsatellite markers was carried out in a G-Storm thermo-cycler (G-Storm, Somerton, UK) using the following temperature cycling conditions; 95°C for 15 min, followed by 35 cycles of 94°C for 30 s, 57°C for 90 s, 72°C for 45 s, followed by a final extension step of 72 °C for 10 min then the samples were held at 4°C. To check for errors in the amplification 30% of the samples were run twice and two negative controls per 96 well plate were included to highlight any contamination should it occur.

Table 2.1. Primer sequences for microsatellite loci used for amplification

Primer	Sequence 5'-3'	Reference
Pv11	F: GTG CTG GTG AAT TAG CCC ATT ATA AG R: CAG AGT AAG CAC CCA AGG AGC AG	(Goodman, 1997)
M11a	F: TGT TTC CCA GTT TTA CCA R: TAC ATT CAC AAG GCT CAA	(Hoelzel et al., 2001)
Hg8.10	F: AAT TCT GAA GCA GCC CAA G R: GAA TTC TTT TCT AGC ATA GGT TG	(Allen et al., 1995)

Fragment analysis:

Fragments were analysed via automated capillary electrophoresis, this technique allows more accurate scoring of allele sizes in comparison to the use of polyacrylamide or agarose gels (Wang et al., 2009).

PCR products were diluted with 10 µl of autoclaved MilliQ water prior to analysis and 2 µl of each of the diluted products were transferred to a 96 well reaction plate containing 40 µl per well of a 400 bp size standard. The size standard was prepared by adding 55

μl of 400 bp size standard (GenomeLab™, Beckman Coulter Ltd, High Wycombe, UK) to 4.6 ml formamide (Sigma-Aldrich, Gillingham, UK). A drop (approximately 0.1 ml) of mineral oil (GenomeLab™, Beckman Coulter Ltd, High Wycombe, UK) was applied on top and the plate briefly centrifuged. Separation buffer (GenomeLab™, Beckman Coulter, High Wycombe, UK) was added to a flat bottomed 96 well plate and both plates loaded into a CEQ™ 8000 Genetic Analysis System (Beckman Coulter, High Wycombe, UK).

Analysis was then completed using CEQ specific software and the resultant fragments assigned homozygous or heterozygous according to the peaks produced. The sizes of the peaks were recorded and analysed graphically in order to assign alleles to each size group.

2.2.2 Statistical analysis:

Datasets were analysed using the open access statistical software package R (R Development Core Team, 2012). Crude odds ratios were calculated for each microsatellite (Pv11, M11a and Hg8.10) by binomial generalised linear models (GLMs) to establish whether homozygosity at a particular locus is a risk factor for neoplasia. Likelihood ratio p values were reported for each GLM. In the event of a significant finding the dataset provided by Dr Acevedo-Whitehouse was analysed in addition to and in combination with the dataset generated by this study. This data set consisted of 270 animals genotyped at the Pv11 locus, 66 of which were UGC positive (Appendix B). Further analysis to identify specific alleles of importance was undertaken by establishing whether the chance of being UGC positive was more likely in animals with certain Pv11 alleles. This was undertaken by calculating the median probabilities from a cumulative binomial distribution at each allele and comparing the number of UGC positive animals to the total number of animals genotyped.

2.2.3 Problems encountered

Contamination was initially encountered and identified at the fragment analysis stage as peaks appearing in the negative control wells. This resulted in the need to repeat the experiments. The problem was overcome by placing the PCR tubes, pipettes and tips in a UV light box for 10 min prior to preparing the multiplex PCR and similarly placing

the 96 well plate, pipettes and tips in the UV light box for 10 min prior to preparing the samples for capillary electrophoresis.

2.3: Results

2.3.1 Spread of alleles

The DNA extracted from the 113 skin samples was of adequate quality for microsatellite analysis. The resultant electropherograms were examined and microsatellite sizes for each of the three microsatellites were recorded and the samples categorised as heterozygous or homozygous. On examination of the peaks those with signal intensity below 1000 relative fluorescence units (RFU) were considered as artefacts. Figure 2.3 illustrates examples of heterozygous and homozygous microsatellites.

Following analysis of the electropherograms each individual allele was plotted on a scatter plot in order to assign allele size groups for each microsatellite; these are illustrated in Figure 2.4. Each microsatellite was identified as being polymorphic in the CSL, with five alleles identified in Pv11 between 176 bp and 184 bp, nine alleles identified in M11a between 136 bp and 152 bp and seven alleles in Hg8.10 between 176 bp and 188 bp.

The most frequent allele identified in Pv11, M11a and Hg8.10 were; allele one (176 bp), allele eight (150 bp) and allele five (184 bp) respectively. The allocated allele number and the frequencies of each allele of the three microsatellites in the 113 animals sampled are illustrated in Figure 2.5. Figure 2.6 illustrates the frequencies of each genotype identified in UGC positive and control animals at the three loci. Appendix A details the resultant genotype of each of the 113 animals in the study.

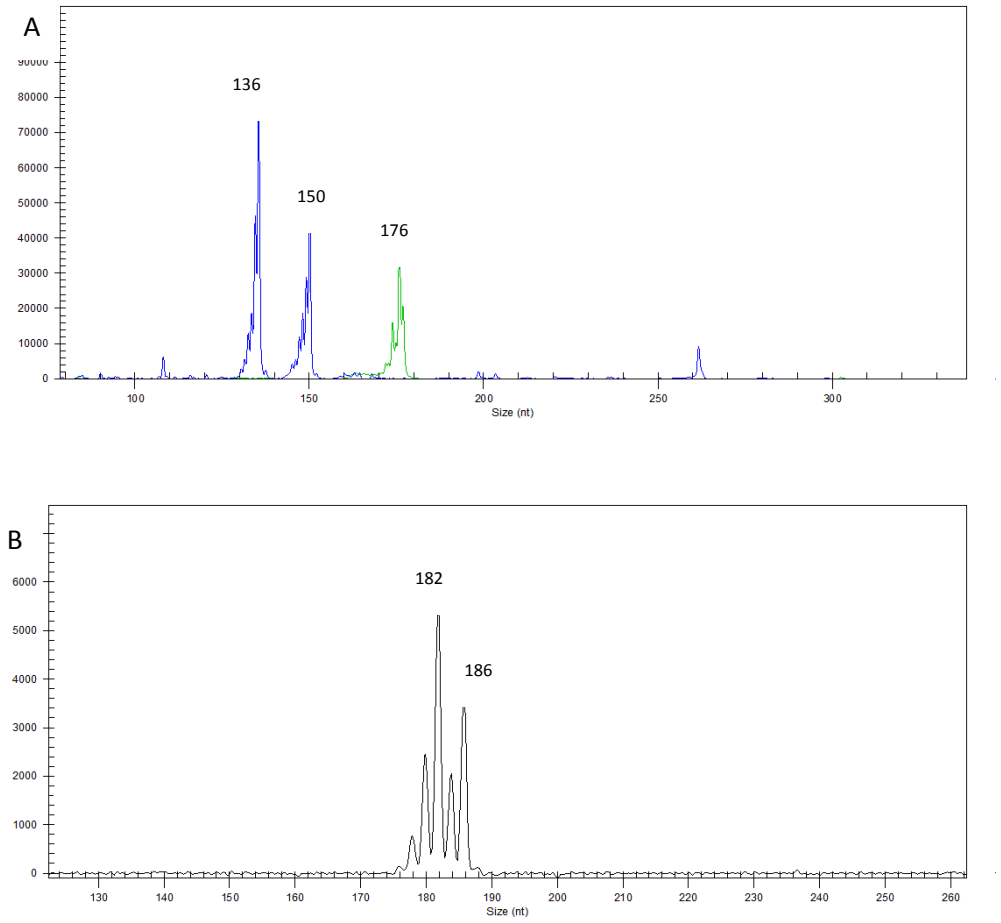


Fig 2.3. Examples of microsatellite electropherograms from CEQ software from two samples. The three microsatellites were included in one multiplex PCR however the Hg8.10 microsatellite (tagged black) was analysed separately due to the allele size range of the microsatellite (176-188bp) overlapping with that of the Pv11 microsatellite (176-184bp). A (sample: 8431(69)): Example of an M11a heterozygote (blue) of allele sizes 136 and 150 and a Pv11 homozygote (green) of allele size 176. B (sample: 9198(21)): Example of a Hg8.10 heterozygote (black) of allele sizes 182 and 186.

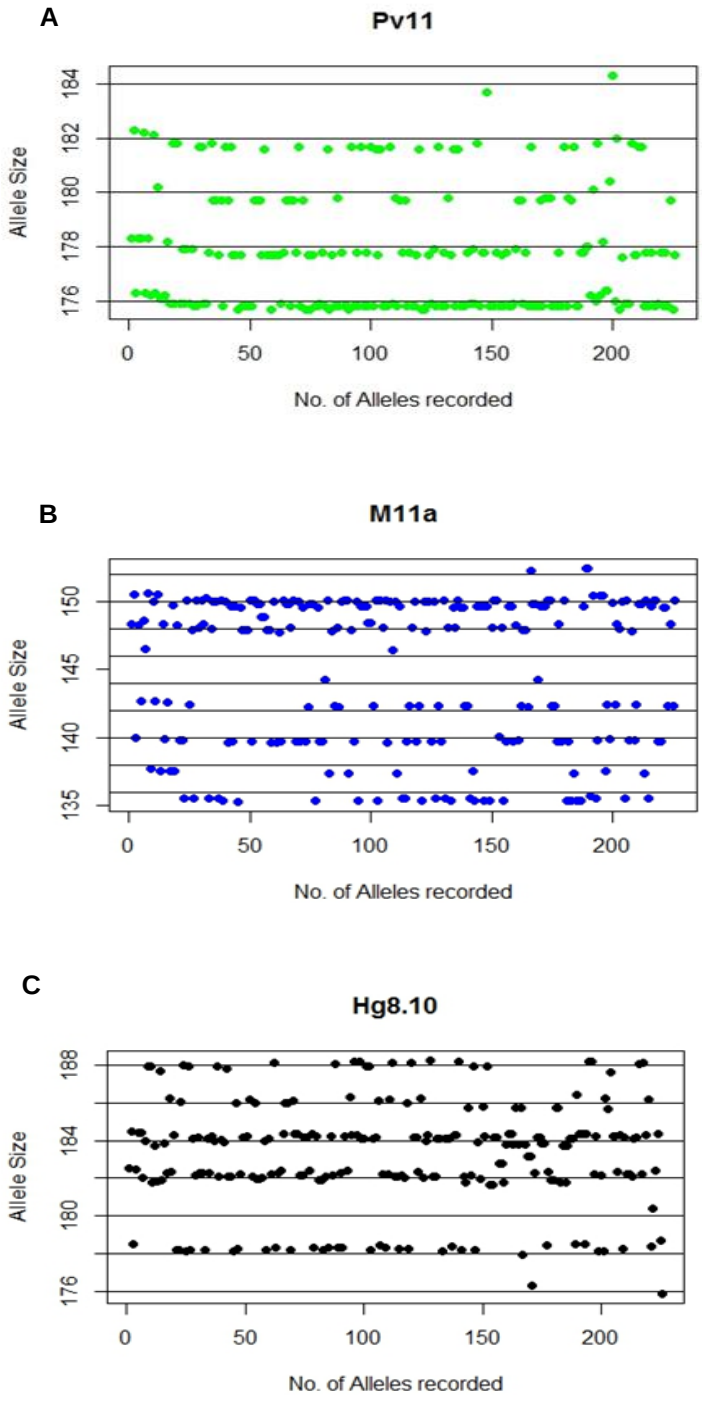
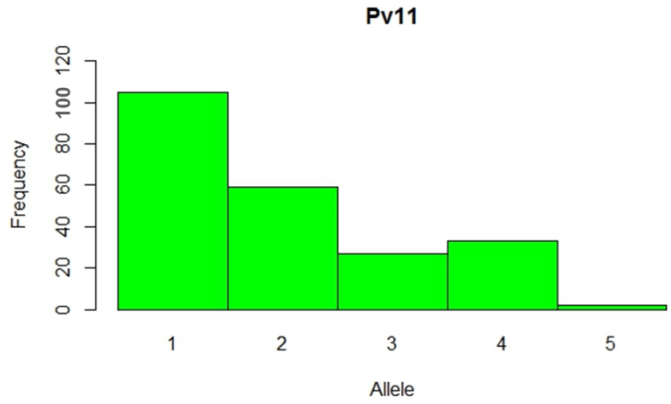
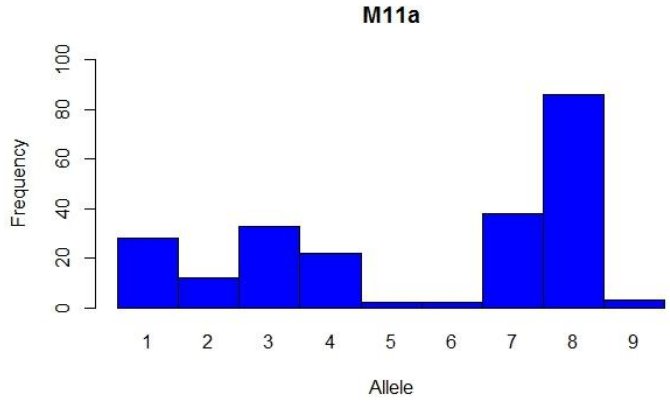


Fig. 2.4 Scatter plots of each microsatellite locus and each of the 226 alleles recorded from the 113 samples analysed. A: Pv11, illustrating five alleles from 176bp to 184bp; B: M11a, illustrating nine alleles from 136bp to 152bp; C: Hg8.10, illustrating seven alleles from 176bp to 188bp

A



B



C

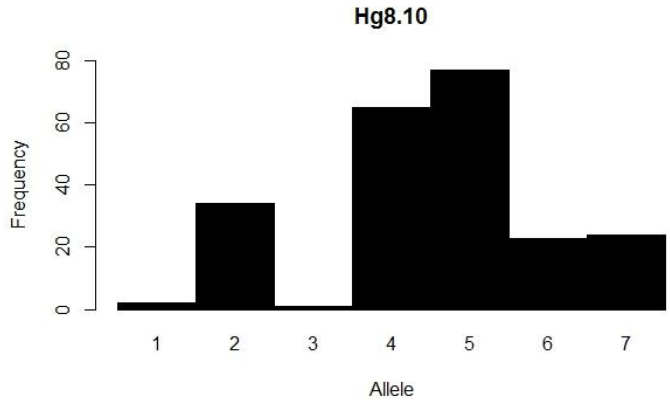


Fig 2.5. Frequencies of the alleles identified in the three microsatellites examined. A: Frequency of the five Pv11 alleles; B: Frequency of the nine M11a alleles; C: Frequency of the seven Hg8.10 alleles

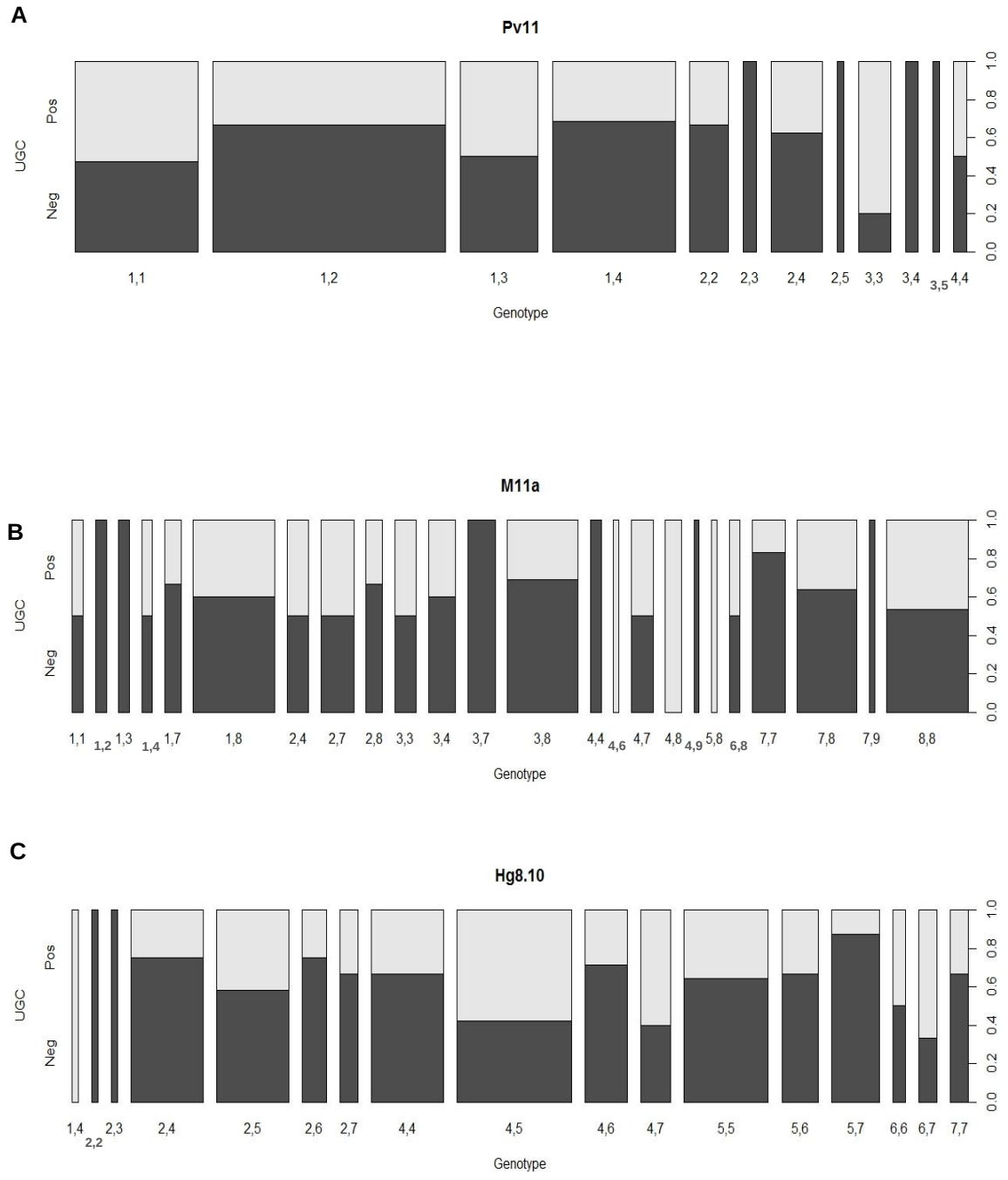


Fig 2.6. Frequency of genotypes identified at the three microsatellite loci examined according to presence (Pos) or absence Neg) of UGC. Bar width corresponds to the proportion of animals of a particular genotype, with the height of dark grey indicating the portion of animals of that particular genotype without UGC and the light grey indicating the proportion animals of a particular genotype with UGC. A: Pv11; B: M11a and C: Hg8.10

2.3.2 Association of homozygosity with UGC

In order to investigate whether homozygosity is associated with the presence of UGC at the three microsatellite loci, GLMs were fitted to the data. Both the M11a and Hg8.10 loci failed to show a significant association with regards to the presence of UGC; however animals homozygous at the Pv11 microsatellite locus were found to be twice as likely to be suffering from UGC, but only at the 10% significance level (Table 2.2). In order to investigate whether this was a small sample size effect as the lower CI for the OR was close to 1.0, the dataset was combined with that supplied by Dr Acevedo-Whitehouse. The GLM was repeated with the expanded dataset and the subsequent results showed that the crude Odds Ratio was 1.62 and the CI was much narrower, giving an increase in significance to p =0.033 (Table 2.2).

Table 2.2 Results of GLM analysis of the three microsatellite loci with regards to the presence of UGC

Microsatellite locus	UGC status	Number of Heterozygotes	Number of Homozygotes	Odds ratio (CI)	P value (LR)
Pv11	Control	54	16	2.00 (0.87-4.6)	0.103
	Positive	27	16		
M11a	Control	52	18	0.99 (0.42-2.37)	0.987
	Positive	32	11		
Hg8.10	Control	49	21	0.80 (0.34-1.89)	0.611
	Positive	32	11		
***Pv11**	Control	183	91	1.62 (1.04-2.58)	0.033
	Positive	60	49		

*Expanded dataset; CI: Confidence Interval; LR: Likelihood Ratio

2.3.3 Association of Pv11 genotype with UGC

Due to the significant relationship identified between homozygosity at the Pv11 locus and the presence of UGC, the various Pv11 genotypes were investigated using the expanded dataset to establish whether a genotype of importance existed. Five comparable alleles were identified in the two datasets. The larger dataset additionally found two extra alleles, only identified in three animals and due to the low frequency of these alleles the animals were removed from the analysis. Figure 2.7A show the frequency of the various allele combinations recorded in both UGC positive and control animals in the expanded dataset. Figure 2.7B illustrates the binomial probability distribution for each genotype, indicating the probability that an animal is UGC positive as opposed to being a control according to a particular genotype. The genotype 3,3 has the highest median probability however its lower confidence interval overlaps with the median probabilities for genotype 2,4 and 4,4 and it was concluded that in this sample there was no statistical evidence that the 3,3 genotype was of any more importance than the other genotypes with regards to the occurrence of UGC. It should be noted however that a number of genotypes identified were present only in small numbers; therefore repeating the analysis with a larger sample population should be undertaken before definitive conclusions are made.

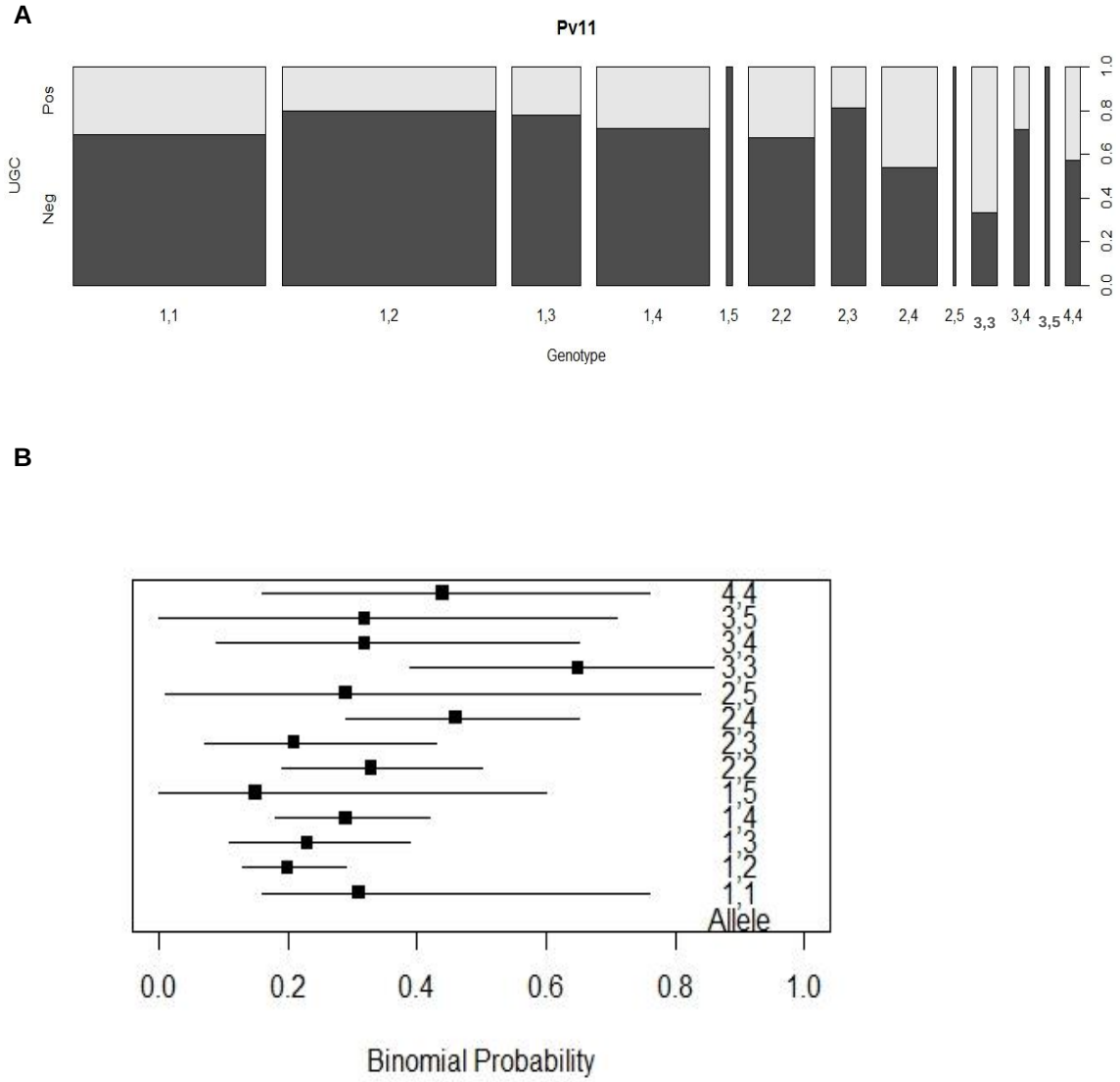


Fig. 2.7 A: Frequency of Pv11 genotypes identified in the expanded dataset according to presence (Pos) or absence Neg) of UGC. Bar width corresponds to the proportion of animals of a particular genotype, with the height of dark grey indicating the portion of animals of that particular genotype without UGC and the light grey indicating the proportion animals of a particular genotype with UGC. B: Binomial probability distributions for each Pv11 genotype identified, showing higher median probability of genotype 3,3 but overlap of the lower confidence interval with the median probabilities for genotype 2,4 and 4,4.

2.4 Discussion

The aim of this study was to investigate the relationship between certain microsatellite loci and the presence of UGC using a sample population free of the confounding factors of sex and age. The study found weak evidence that animals homozygous at the Pv11 locus are around twice as likely to be suffering from UGC as animals that are heterozygous at this locus (crude OR: 2.00, CI: 0.87-4.6, p=0.103). Although only significant at the 10% level, in light of the odds ratio identified (and the lower CI being close to 1.0) along with the previous findings by Acevedo-Whitehouse (unpublished), it was decided that further investigation was necessary to improve the power of the study. The data set therefore was combined with that provided by Dr Acevedo-Whitehouse. Analysis of the combined dataset resulted in a strengthened relationship between homozygosity at the Pv11 locus and the presence of UGC, however this re-introduced confounding factors of sex and age (crude odds ratio: 1.62 CI: 1.04-2.58, p=0.033). No significant relationships were found with any particular Pv11 allele or with homozygosity at the M11a locus.

To verify the trend identified here and to look in greater depth for potential Pv11 alleles of importance it is necessary to expand the single sex/age data set significantly. The present study is nested within a wider epidemiological study being carried out over a number of years by the Sea Lion Cancer Consortium (SLiCC) and ultimately the aim is to undertake a sex and age matched analysis in a larger sample population than was possible in the present study. Power calculations that have been carried out in order to design the larger case-control study have shown that a minimum of 100 UGC positive animals along with 200 controls are necessary to detect a true difference of the expected magnitude (Hall and Gulland, 2011).

Understanding what the result of this study is telling us is difficult. The association between Pv11 homozygosity and cancer may simply reflect inbreeding, resulting in reduced heterozygosity and therefore reduced fitness (Osborne et al., 2011, Keller and Waller, 2002). Homozygosity and reduced fitness has been noted in CSL before, where it was identified that more inbred individuals have longer recovery time from disease (Acevedo-Whitehouse et al., 2003). However with previous work suggesting that the relationship between inbreeding and cancer in the CSL was driven by the Pv11

microsatellite locus there is an implication that this microsatellite holds greater importance in the development of the disease (Acevedo-Whitehouse, unpublished). It is therefore possible that the microsatellite is linked to a fitness related gene (Osborne et al., 2011, Hansson et al., 2004, Balloux et al., 2004). The CSL genome has been sequenced (Dinsdale, 2011), however it is low coverage at present and consists of many unassembled short reads. Therefore it is not possible to pinpoint the location within the genome of a particular marker, making the identification of associated genes problematic. Further work has been carried out to identify the location of Pv11 using comparative genomics and molecular techniques and this is covered in Chapter 3.

Similar situations have been reported in relation to homozygosity and disease susceptibility in both humans and animals and include studies into the pathology associated with hookworm infection (*Uncinaria* spp.) in CSL pups and New Zealand sea lion (*Phocarctos hookeri*) pups (Acevedo-Whitehouse et al., 2006, Acevedo-Whitehouse et al., 2009) and mortality associated with invasive bacterial infection in children (Lyons et al., 2009). In the CSL pups the study involved the genotyping of 181 pups at 13 microsatellite loci and of those with hookworm attributed lesions (n=130) it was identified that anaemia was associated with homozygosity at a particular microsatellite locus (Hg4.2) (Acevedo-Whitehouse et al., 2006). Similarly genotyping of 39 New Zealand sea lion pups at 22 microsatellite loci revealed that homozygosity at a single locus (ZcCgDh3.6) was associated with anaemia (Acevedo-Whitehouse et al., 2009). In humans an association was found concerning homozygosity at five microsatellite loci (out of 134 genotyped) in children succumbing to bacterial infections in Kenya. Interestingly the strength of the association was seen to change between type of infection (gram positive or gram negative) and also age class (Lyons et al., 2009). This highlights the importance of both accurate identification of cause of death or morbidity along with careful matching in a case-control study when investigating these associations.

In humans, genome wide association studies (GWAS) using both microsatellites and single nucleotide polymorphisms (SNPs) as markers have been employed in investigating disease with a suspected genetic component especially those with a familial root (Lyons et al., 2009, Chung et al., 2010, Burton et al., 2007). A previously

mentioned example was that of studies into type 2 diabetes (Grant et al., 2006). Various cancers have also been investigated in this way including prostate cancer. This led to the identification of variants of a particular microsatellite allele on chromosome eight in humans being found to be associated with the disease (Amundadottir et al., 2006, Gulcher, 2012). GWAS have also been carried out in animals both to look for candidate genes of importance in disease for example in canine atopic dermatitis and canine systemic lupus erythematosus (Tengvall et al., 2013, Wilbe et al., 2010) and, largely for economic reasons, in looking for genes of importance in race horse performance or productivity in dairy cattle (Tozaki et al., 2010, Meredith et al., 2012).

The multi-factorial aetiology of neoplasia indicates that the relationship identified here between homozygosity at the Pv11 locus and UGC is unlikely to be the full story with regards to the development of the disease. The odds ratio reported is considered crude as it does not take into account other factors previously identified as being linked to the occurrence of UGC. These include persistent organic pollutants and herpesvirus (Buckles et al., 2006, Ylitalo et al., 2005) and it is possible that the strength of the association between Pv11 and UGC may increase once these additional exposures have been accounted for. This will be explored further in Chapter 7. In spite of the small sample size the identification of a microsatellite marker with a significant relationship to the occurrence of cancer does offer a starting point for investigating a genetic basis of the disease.

Chapter 3

Identification of the genetic location of the Pv11 microsatellite marker in the California sea lion (*Zalophus californianus*) - a non-model wild species

3.1 Introduction

Genome wide association studies (GWAS) are used to investigate a disease with a suspected genetic component. The markers (commonly SNPs or microsatellites) used in these studies assist in the identification of regions of interest in the genome known as quantitative trait loci (QTL) (Meredith et al., 2012, Zhang et al., 2012). For the identification of a candidate gene it is presumed that linkage disequilibrium is occurring (Zhang et al., 2012, Wray et al., 2008). Linkage disequilibrium (LD) describes the non-random association of alleles, where alleles of different loci are found together more often than expected, as opposed to linkage equilibrium (LE) where alleles of different loci are randomly associated and occur together at no greater frequency than would be predicted (Goldstein and Weale, 2001, Lewontin and Dunn, 1960, Geiringer, 1944, Nicholas, 2010c). Identification of markers in LD with specific genes allows linkage mapping, where loci are mapped according to recombination frequency instead of distance apart on the chromosome (Wong et al., 2010, Mellersh et al., 1997, Werner et al., 1999, Nicholas, 2010b).

If, following a GWAS, a particular marker shows an association with a trait or disease, the information can be used to identify a QTL. QTLs are commonly very large and can be over 20 cM encompassing many genes (Miles and Wayne, 2008, Meredith et al., 2012, Flaherty et al., 2005). The identification of a potential gene candidate responsible for the trait or disease of interest may also be possible. However a number of genes may be responsible for a trait and the effect of a single gene alone may be quite small, thus complicating the understanding of a genetic basis of a condition (Meredith et al., 2012, Miles and Wayne, 2008, Stranger et al., 2011, Flaherty et al., 2005, Wray et al., 2008). In addition an assembled genome covering the region of interest is necessary in identifying gene candidates (Osborne et al., 2011). The previous chapter demonstrated that animals homozygous at the Pv11 locus were almost twice as likely to be suffering

from UGC, than those that were heterozygous. It is therefore possible that Pv11 is marking a QTL and identifying its location within the genome and genes within its vicinity may yield information concerning the aetiology of UGC, this in turn could indicate pertinent routes of further investigation. A limitation of the present study however, is the lack of an assembled genome for the CSL or indeed any other pinniped (Osborne et al., 2011). This complication however may be partly overcome by comparing the genomes of closely related species where conservation of segments of the genome is seen to occur (Osborne et al., 2011, Ferguson-Smith and Trifonov, 2007, de Grouchy et al., 1978, Dawson et al., 2006). A predicted map of genetic markers has been created based on chromosomal synteny for pinnipeds using sequence alignments of phylogenetically related members of the order carnivora; the domestic dog (*Canis lupus familiaris*), domestic cat (*Felis catus*) and giant panda (*Ailuropoda melanoleuca*) along with using the dog genome as a chromosome scaffold (Osborne et al., 2011). Osborne et al (2011) went on to investigate potential gene candidates associated with the microsatellites Hg4.2 and ZcCgDh3.6. As discussed in Chapter 2 homozygosity of Hg4.2 and ZcCgDh3.6 were noted as being important in the pathology of hookworm infection in in CSL and New Zealand sea lion pups respectively, specifically in relation to the occurrence of anaemia (Acevedo-Whitehouse et al., 2009, Acevedo-Whitehouse et al., 2006). This finding suggested that these microsatellites were in LD with genes involved in this pathology and the predicted microsatellite map along with analysis of the regions of the dog genome were used to suggest potential candidate genes (Osborne et al., 2011). Genetic conservation therefore offers a starting point in identifying the location of Pv11 in the CSL. The study undertaken by Osborne at al., (2011) did include Pv11 and placed it on chromosome 28 of the canine genomic scaffold; however it did not examine genes in the vicinity. The present study aimed to use both comparative genomics and molecular methods to confirm the location of Pv11 in the CSL genome and if possible, to identify genes within this genomic location.

[1]3.2 Materials and Methods

3.2.1 Comparative genomics

In the absence of an assembled CSL genome other methods were undertaken to establish the location of the Pv11 microsatellite; these included the implementation of molecular methods and comparative genomics. A short region of sequence flanking the Pv11 microsatellite consisting of 153bp was analysed using the NCBI Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). The analysis identified a region in the *HPSE2* gene on chromosome 28 in the dog genome with 91% nucleotide identity over 83% of sequence. Subsequent alignment of this region in the horse, dog and pig enabled the design of primers to amplify a larger region of 2kb across the Pv11 microsatellite. This larger gene fragment was found to be clearly orthologous to intron 9 of the *HPSE2* gene (Hammond, unpublished).

Further analysis utilizing comparative genomics was then carried out confirming that this gene is conserved in mammals (Hammond, unpublished). The *HPSE2* gene is large in all mammals and in the dog spans over 630kb. It is comprised of 12 exons separated by large intronic regions, with intron 9 being 101kb in size (NCBI Gene ID: 486831). The *HPSE2* gene will be discussed in more detail in section 3.4.

3.2.2 Southern Blot

To provide supporting evidence for the location of Pv11 within the *HPSE2* gene a southern blot was carried out.

Sample selection

To perform the Southern blot, genomic DNA from harbour seal (*Phoca vitulina*) and CSL were used. These species are descended from a common canine ancestor and therefore conservation of the genetic structure is expected. The advantage of using both species in the Southern blot is that it allows the confirmation of genetic conservation.

[1] The comparative genomic work was carried out by Dr John Hammond prior to the start of my PhD. The Southern Blot was undertaken in collaboration with Dr John Hammonds Lab at The Compton Laboratory, The Pirbright Institute, Compton, UK.

The Southern blot was undertaken using the DIG Luminescent Detection Kit for Nucleic Acids, (Roche Applied Science, Mannheim, Germany). Southern blots allow the detection of specific DNA sequences along with the potential detection of multiple homologous genes in a genome should they occur. In order to achieve this the digoxygenin (DIG) system relies on DNA probes labelled with DIG which following hybridisation to target DNA sequences, are bound by an anti-DIG antibody which itself has the enzyme alkaline phosphatase (AP) attached to it. The addition of a chemiluminescent AP substrate subsequently results in the detection of the hybridised probe. The process requires a number of steps; (1) DNA extraction, (2) restriction digestion and DNA separation, (3) gel preparation and blotting, (4) preparation of probes, (5) hybridization and (6) detection. The steps undertaken are detailed in full in Appendix D.

Two probes were employed in the study, therefore prior to the southern blot two identical restriction digests per DNA sample were carried out. In order to undertake a restriction digest restriction enzymes (RE) are used. RE are derived from bacteria and make up part of a bacterium's defence mechanism against viral infection reviewed by Nicolas, 2010 (Nicholas, 2010b). A particular RE has the ability to "cut" DNA at a specific sequence. The particular sequence may occur more than once throughout the genome therefore the DNA is subsequently digested into a number fragments (Nicholas, 2010b), for example digestion of the human genome with the RE *EcoRI* (from *Escherichia coli)* results in approximately 800,000 fragments (Chen et al., 2008). The frequency of sites recognised by RE throughout the genome depends on the size of their recognition sequence. In the human genome a recognition sequence for a RE specific to a 6bp sequence will occur approximately every 4kb whereas one with a 4bp recognition sequence is expected to occur more frequently (approximately every 250bp) (Griffiths et al., 2000). There have been over 3600 RE identified of which around 580 are commercially available (Roberts et al., 2005).

The restriction enzymes BamHI (New England Biolabs Hitchin, UK) and HindIII (New England Biolabs Hitchin, UK) were chosen as they were identified as suitable due to their ability to cut the DNA into the larger fragments required due to the large size of the intron 9 where Pv11 is expected to be situated (Hammond, unpublished). This

increased the chances that the sequences targeted by the probes wouldn't be on different fragments of DNA, thus allowing a greater chance of hybridisation of the probes to the same DNA section.

The two probes used in the Southern Blot were called probe A and probe B. Probe A was 520bp in size and incorporated 22bp of exon 9 of the *HPSE2* gene whereas probe B was 1kb in size and flanked the Pv11 microsatellite. The position of the two probes is illustrated in Figure 3.1.

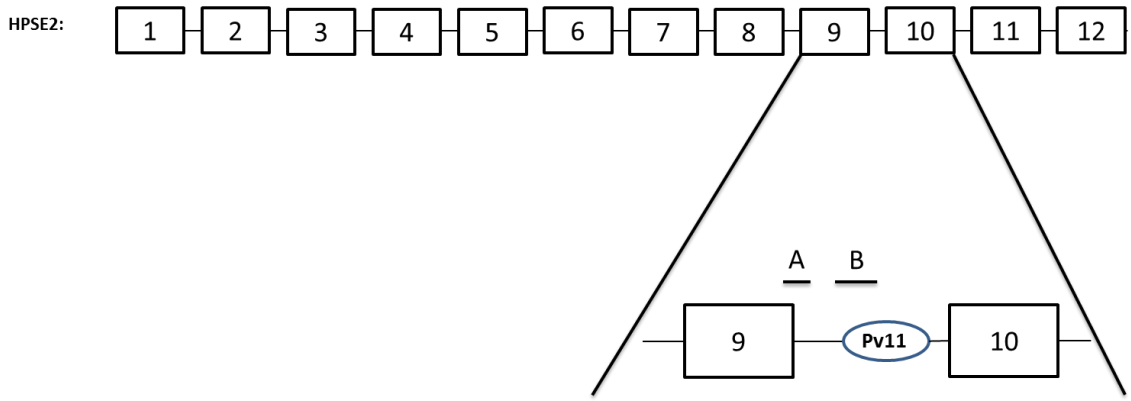


Fig 3.1: Structure of the *HPSE2* gene is conserved amongst mammals and consists of 12 exons separated by large intronic regions. The pop out window illustrates the position of the two probes (A and B) employed in the southern blot. Probe A being ~500bp in size and incorporating 22bp of exon 9 and probe B being ~1kb in size and flanking the Pv11 microsatellite.

In order to make the two probes, 50 µl PCR's were carried out using CSL genomic DNA as a template and degenerate PCR primers supplied by Sigma-Aldrich (Gillingham, UK). Degenerate primers allow the amplification of DNA fragments via PCR in cases where the actual sequence is unknown and are designed by examining DNA sequences of a genetically similar species (Kwok et al., 1994, Lang and Orgogozo, 2011). In the case of the present study the primers were designed using the dog, horse, cow and harbour seal genome as illustrated in Figure 3.2 (Hammond, unpublished). The sequences of the primers used to make the probes are detailed in Table 3.1. The 50 µl reactions consisted of 10x NH4 buffer (Bioline, London, UK), 1.25 µl 50 mM $MgCl_2$, 0.5 µl 10 mM dNTP (Invitrogen, Paisley, UK), 1 µl of forward and reverse primer from 10 µM stock solutions, 0.5 µl (2 units) Bio-X-ACT short DNA

polymerase (Bioline, London, UK), 38.75 µl of RNase free water (Qiagen, Crawley, UK) and 2 µl (124 ng/µl) of DNA template. The reaction cycle for probe A was as follows 95°C for 1 min, followed by 35 cycles of 95°C for 1 min, 59°C for 1 min, 72°C for 2 min, then 72°C for 15 min before being stored at 4°C. For probe B the same reaction cycle was used with the modification of the annealing temperature being raised to 64°C. The products were resolved on 1% agarose gels and bands of appropriate size extracted and purified using QIAquick gel extraction kit (Qiagen, Crawley, UK) as per manufacturer's instructions. The product was quantified by running 5 µl of the purified product alongside three lambda DNA size markers: 25 ng, 50 ng and 100 ng (Promega, Southampton, UK) on a 1% agarose gel.

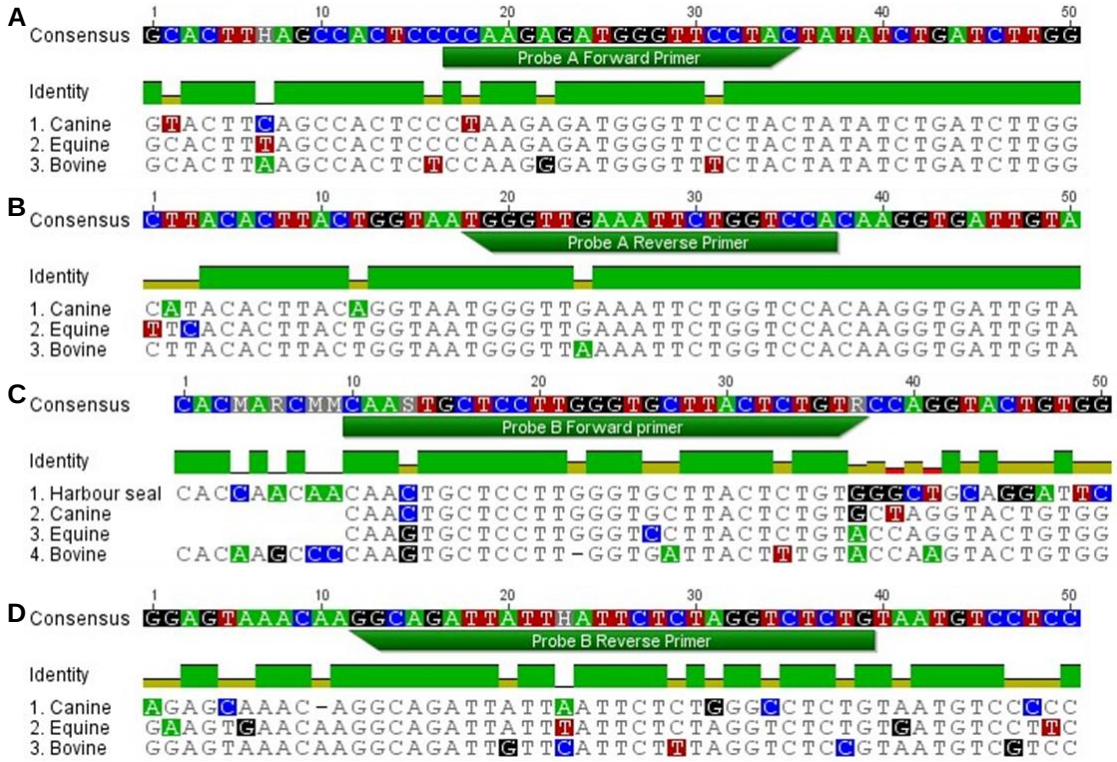


Fig 3.2. Sequence allignments from the dog, horse, cow and harbour seal of four regions in the *HPSE2* gene used in order to design primers for southern blotting. These alignments illustrate where the degenerate sites arise. A and B illustrate the forward and reverse primers used to make probe A; C and D illustrate the forward and reverse primers used to make probe B.

Table 3.1. Primer sequences used to make probes for Southern blotting

Probe	Sequence 5'-3'	Reference
Exon (probe A)	F: CYA AGA GAY GGG TTC CTA C R: TGG GTT YAA ATT CTG GTC CA	(Hammond, unpublished)
Pv11 (probe B)	F: CAA CTG CTC CTT GGG TGC TTA CTC TGT G R: GGC AGA TTA TTW ATT CTC TYG GRC TCT G	(Hammond, unpublished)

3.3 Results

The two DNA probes (A and B) utilised in the Southern blot hybridised to the same CSL genomic DNA fragment and was replicated in the harbour seal (Figure 3.3). This, alongside the previous comparative genomic investigation (Hammond, unpublished) confirmed that the Pv11 microsatellite is located within intron 9 of the CSL HPSE2 locus.

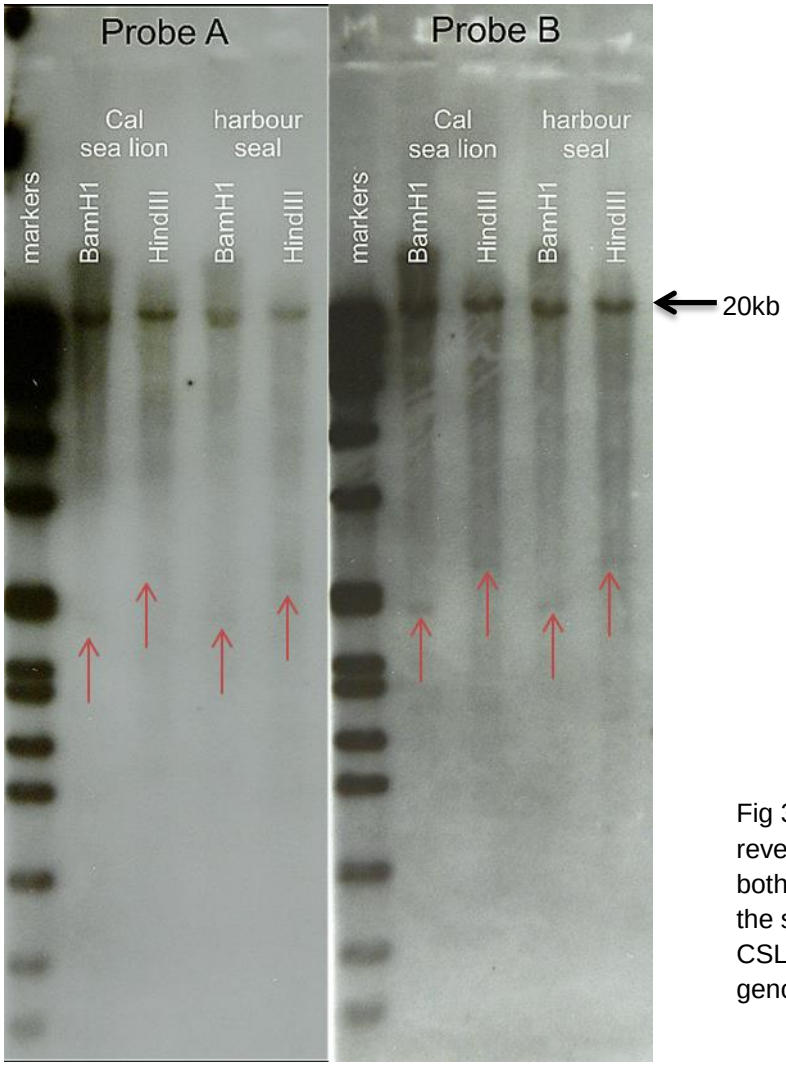


Fig 3.3. Southern blot revealing hybridisation of both probes (A and B) to the same location in the CSL and harbour seal genome.

3.4 Discussion

Genotyping of the Pv11 microsatellite revealed that animals homozygous at this marker were approximately twice as likely to suffer from UGC as those that were heterozygous. Analysis using comparative genomics suggested the location of Pv11 was within the *HPSE2* gene in the CSL, a conserved location in mammals. This genomic location was then verified by Southern blot. Examination of published genomes of a variety of species in the NCBI database revealed a number of genes in the vicinity of *HPSE2*. It is possible that Pv11 is marking a QTL and as discussed earlier these can cover large genomic regions containing a number of genes, thereby making the identification of the gene or genes responsible for a particular disease or trait difficult. However the large size of the *HPSE2* gene across all species examined suggests it has an important role, thus making it a probable gene candidate involved in UGC in the CSL; hence this gene was investigated further.

The *HPSE2* gene was discovered by McKenzie et al., (2000) and the protein, HPA2, it encodes was found to share approximately 35% amino acid sequence identity over the coding regions with the protein heparanase 1 (HPA1) encoded by the heparanase gene (*HPSE*) (McKenzie et al., 2000). *HPSE* has been the subject of much research due to its involvement in neoplasia (Vlodavsky et al., 2005, Vlodavsky et al., 1999); therefore a gene sharing sequence similarity is of potential clinical interest. In comparison to *HPSE*, research into *HPSE2* is still in its infancy and only a handful of published papers exist regarding its potential role (Levy-Adam et al., 2010, Pang et al., 2010, Peretti et al., 2008, Mahmood et al., 2012, Marques et al., 2012, de Moura et al., 2009, Giordano, 2008, McKenzie et al., 2000, Zhang et al., 2013, Daly et al., 2010). In light of the lack of information regarding *HPSE2* along with the sequence similarity between HPA1 and HPA2, the function and activity of *HPSE* and its protein HPA1 will be discussed first.

HPA1 has enzymatic activity; it is an endo-β-glucuronidase which acts on the substrate heparan sulfate, breaking it down into smaller fragments reviewed by Barash et al., 2010 (Barash et al., 2010). The importance of this action (in terms of neoplasia) is the effect the enzyme has on heparan sulfate proteoglycans (HSPG) which are an important constituent of the basement membrane (BM) and extracellular matrix (ECM). Cleavage of heparan sulfate chains from their proteoglycan core results in loss of cellular integrity

thereby permitting cellular invasion and subsequent metastasis (Edovitsky et al., 2004, Shafat et al., 2008, Barash et al., 2010, Levy-Adam et al., 2010). This action additionally causes the release of bioactive molecules such as cytokines and growth factors attached to the heparan sulfate which are involved in cell growth and angiogenesis (Boyd and Nakajima, 2004, Barash et al., 2010, Ostrovsky et al., 2009).

Increased expression of *HPSE* mRNA and HPA1 protein production has been noted in a number of cancers affecting a variety of body tissues including colon, bladder, breast and pancreas amongst others (Vlodavsky et al., 2005, Peretti et al., 2008, Zhao et al., 2009). In addition HPA1 has been identified as a potential diagnostic and prognostic marker with high levels indicating a poor prognosis and reduced survival time (Ilan et al., 2006, Davidson et al., 2007, Shafat et al., 2008, Zhao et al., 2009).

The majority of the published research has concentrated on *HPSE's* role in cancer metastasis; however other roles of the enzyme have been explored. These include in embryo implantation in the uterus and inflammation (Rodrigues et al., 2011, D'Souza et al., 2008). In addition to HPA1 enzymatic action in remodelling of the ECM it also has been found to have a pro-adhesive role due to clustering of HSPG, this action is pH dependent (Gilat et al., 1995, Levy-Adam et al., 2010). HPA1 is initially expressed as an inactive pro-enzyme, 65 kDa in size and proteolytic processing by protease enzymes such as cathepsin L is required to convert it into an active enzyme. The 65 kDa pro-enzyme is cleaved into two parts by removing a 6 kDa linker segment. The two resultant sub units (of 8 kDa and 50 kDa) join together to form the active enzyme that is responsible for HSPG degradation (Arvatz et al., 2011).

HPSE is found to be conserved amongst mammals, however in invertebrates examined, the gene, although present, shows similarity within other members of the class insecta rather than between classes (Shaik et al., 2012, Vlodavsky et al., 1999). In *HPSE2* however although the gene is identified as conserved in mammals, unlike *HPSE,* it has not yet been identified in invertebrates (Pang et al., 2010). *HPSE2* is a large gene consisting of 12 exons and is approximately 630 kb (NCBI Gene ID: 486831) and 776 kb (Pang et al., 2010) in the canine and human respectively. Alternative splicing of exons three and four in humans results in four putative proteins containing 480, 534, 538 and 592 amino acids (Daly et al., 2010). Further sequence analysis found that the

similarity in HPA1 and HPA2 sequences was particularly strong across the two subunits that join to make up the active enzyme of HPA1 (50 kDa and 8 KDa), however the linker region, which is cleaved during proteolytic processing, was not well conserved (McKenzie et al., 2000). Further to this it was found that mRNA expression patterns of *HPSE* and *HPSE2* differed in normal tissues.

HPSE is expressed in greater levels in placenta and lymph node whereas *HPSE2* expression is found to be absent or low in these tissues, but is higher in mammary gland, prostate, small intestine, testis and uterine tissue along with developing and adult urinary bladder and kidney (Daly et al., 2010, McKenzie et al., 2000). Expression of both genes was also seen in different regions of the brain (McKenzie et al., 2000, Daly et al., 2010). When tissue affected by neoplasia was examined, there was also a disparity in the expression pattern of these two genes, with one study reporting high levels of *HPSE* expression in all tumour tissues examined (breast, colon, lung, prostate, ovarian and pancreatic), and low levels of *HPSE2* expression in the same tissues except for in the pancreas where it was significantly higher (McKenzie et al., 2000).

Although expression profiles of *HPSE2* mRNA have been carried out using many tissues the results of these do not necessarily provide information regarding the presence of the protein (Huang et al., 2011). A few studies have investigated the presence of the protein. These immunohistochemical and immunocytochemical studies used a polyclonal antibody raised against human HPA2 (with the ability to detect three of the reported isoforms) to identify the presence of the protein in ovarian cancer (de Moura et al., 2009), head and neck tumours (Levy-Adam et al., 2010), colorectal cancer (Peretti et al., 2008), cancer of the cervix (Marques et al., 2012) and gastric cancer (Zhang et al., 2013). All studies identified that HPA2 was up-regulated in the neoplastic regions and Table 3.2 sums up the published information available. Marques et al., (2012) additionally found an increase in HPA2 expression with an increase in the severity of the lesion. However, the study undertaken by de Moura et al., (2009), investigating HPA2 expression in ovarian neoplasia, found that there was no difference in expression in malignant compared to benign tissues, although differences did occur with stage of differentiation. In this case the authors concluded that HPA2 was involved in tumour expansion but did not definitively conclude how.

Table 3.2. Summary of studies into the presence of HPA2 in various human tumours

Tumour site	hpa2 presence	Reference
Head and neck tissue	Increased in tumour tissue; undetected in adjacent normal epithelium	(Arvatz et al., 2011, Levy-Adam et al., 2010)
Breast (study into hpa2 in lymphocytes)	High in lymphocytes in patients; Low in controls	(Theodoro et al., 2007)
Ovary	High in neoplastic epithelial tissue low or absent in normal	(de Moura et al., 2009)
Cervix	Increased presence with increased severity of cancer, lower in normal tissue	(Marques et al., 2012)
Stomach	Increased in cancer tissue, low in adjacent normal tissue	(Zhang et al., 2013)
Colon	High in carcinoma affected tissues, low in non-neoplastic tissue	(Peretti et al., 2008)

Although the majority of studies identifying HPA2 have involved human tissue the protein has been identified in rats in a study investigating intervertebral disc degeneration (Oliveira et al., 2013). Further to this *HPSE2* homologues have been reported in a number of species (NCBI, HomoloGene: 19680).

It was initially believed that *HPSE2* had a similar action to *HSPE*, however (Levy-Adam et al., 2010) published a study which took steps towards characterising the gene and discovered important differences with *HSPE*. Unlike HPA1, HPA2 was found not to exhibit enzymatic activity and also had a stronger affinity to heparin and heparan sulfate than HPA1. This action led the authors to postulate that HPA2 may in fact work against HPA1, potentially inhibiting its action. HPA2 expression was investigated in head and neck carcinoma and it was deduced that metastasis was higher in cases where HPA2 expression was lower (Levy-Adam et al., 2010). Additionally, disease recrudescence was longer where HPA2 expression was higher, this finding was

replicated in studies into gastric cancer where better prognosis was seen in cases of higher HPA2 expression (Zhang et al., 2013, Levy-Adam et al., 2010).

Similarly to the investigations into *HPSE* and neoplasia further potential roles were noted with regards to *HPSE2*. For example, its increased expression in the uterus suggests a role (as with *HPSE*) in pregnancy, however further work is required to confirm this (D'Souza et al., 2008). In 2010 another development in the *HPSE2* story was discovered when researchers identified a role of *HPSE2* in a genetic disorder, urofacial syndrome (or Ochoa syndrome). Urofacial syndrome (UFS) (OMIM#236730) is an autosomal recessive condition occurring in humans that has been linked to the *HPSE2* gene (Daly et al., 2010, Pang et al., 2010, Mahmood et al., 2012). The condition is characterised by a number of clinical symptoms relating to dysfunctional urination including recurrent urinary tract infections, dysuria and incontinence (Pang et al., 2010). If left untreated the defects affecting the urinary tract can cause the condition to progress to renal failure. The most notable symptom of UFS is a facial grimace made when the patient attempts to smile (Pang et al., 2010). A number of mutations in *HPSE2* have been attributed to the condition including in frame whole exonic deletions and frameshift mutations due to insertions or deletions of base pairs (Daly et al., 2010, Pang et al., 2010). It is noted that deletions of exon three are of particular importance in cases of UFS (Daly et al., 2010). Additionally UFS has also occurred with no identified mutation to *HPSE2* suggesting that it is a heterogeneous condition (Woolf et al., 2013). UFS is presently considered a rare condition and Mahmood et al., (2012) state that so far there have been only 18 published cases. However, diagnosis is difficult partly due to variation in severity of clinical presentations that result in it not being recognised, the condition may therefore be more common than is presently reported (Mahmood et al., 2012).

The uncertainty surrounding the role of *HPSE2*, especially in its relation to the inhibition or promotion of neoplasia, presents the question of whether this gene has oncogenic activity or is in fact a tumour suppressor gene. The location of the Pv11 microsatellite within the *HPSE2* gene of CSL and its link with the homozygous state and UGC, offers another avenue of investigation in characterising this gene.

Chapter 4

Limited genetic instability is present in lower genital tract tissue from California sea lions (*Zalophus californianus*) with and without urogenital carcinoma

4.1 Introduction

Neoplasia is essentially a genetic disease; its occurrence being a consequence of genetic alterations that result in the loss of control of cell division (Hahn and Weinberg, 2002, Collins et al., 1997). When this occurs, cells can proliferate uncontrollably and in the case of metastatic neoplasia, spread to parts of the body which are distant from the primary lesion (Vile and Morris, 1992, Adkinson and Brown, 2007).

More than one event affecting the genetic code of the cell is necessary for the development of neoplasia (Hahn and Weinberg, 2002, Land et al., 1983) and in a study regarding endometrial neoplasia it was proposed that six genetic alterations were necessary for tumour development (Peiffer et al., 1995). However the true number of alterations in a particular cancer remains undetermined and is almost certainly tumour dependent. Genetic alterations may involve a hereditable component leaving certain animals predisposed, but the majority occur due to spontaneous mutations in somatic cells which are left uncorrected by the cells safety mechanisms (Bunz, 2008, Hsieh and Yamane, 2008, Srivastava and Grizzle, 2010). The rate of mutation of DNA in higher eukaryotic organisms is much less than that identified in lower organisms. In RNA viruses for example mutations can be as frequent as one per genome per replication as reviewed by Drake et al., 1998 (Drake et al., 1998). This in comparison to a rate of 2 to 30×10^{-7} mutations per cell division identified in a study of B lymphoblastoid cells from normal human donors (Araten et al., 2005). Mutations in DNA can occur as a result of factors within the cell, for instance by-products of respiration; however mutations can also occur due to exposure to external factors such as radiation or cigarette smoke, with one study identifying a 56% increase in mutation frequency in T-lymphocytes in smokers compared to non-smokers. Increases in mutation rate increase the risk of a deleterious outcome (Beebee and Rowe, 2008, Malkin, 1995, Ding et al., 2011, Cole et al., 1988).

Mutations within the genome can occur on a large scale, to the extent that the chromosome number may be altered from that normally observed for the species (Gordon et al., 2012). In neoplasia this has been seen in the neoplastic cells of canine transmissible venereal tumour (CTVT) and the more recently reported Devil facial tumour disease (DFTD) in the Tasmanian devil (*Sarcophilus harrisii*) (McAloose and Newton, 2009, Murchison, 2009, Thomas et al., 2009). In these cases the tumour cells themselves act as infectious agents. Alterations in DNA can occur on a smaller level than those affecting whole chromosomes. Translocations of genes can have a significant effect, for example, if the genes are moved to a location where they are placed under different promoter control, as in the case of Burkitts lymphoma (Madisen et al., 1998). In this condition translocation of the *MYC* gene, a gene involved in cell division, is placed under control of a promoter which increases its expression (Madisen et al., 1998, Adkinson and Brown, 2007). DNA mutations can also occur at the level of the nucleotides where point mutations in the form of additions, deletions or substitutions can affect the translation of a gene product (Beebee and Rowe, 2008, Bunz, 2008).

Microsatellite instability (MI) and Loss of Heterozygosity (LOH) are two forms of genetic instability. LOH describes the situation where loss of an allele occurs rendering a previously heterozygote locus homozygote (Dietmaier et al., 1999, Thiagalingam et al., 2002) and is discussed below. MI refers to the situation where alterations in microsatellites occur during DNA replication and result in expansion or contraction of the repeat unit. These changes can result in frameshift mutations and subsequent erroneous gene transcription (Sourvinos et al., 1997, Oda et al., 2002, Yamamoto et al., 1997, Imai and Yamamoto, 2008). MI has been noted to be a result of a mutation in one of the genes of the mismatch repair pathway (*MMR*). The *MMR* is a complex of genes that offer the cell protection against mutations (Hussein and Wood, 2002, Aquilina and Bignami, 2001). Mutations occurring in the *MMR* genes can render their protective mechanisms redundant and promote the development of neoplasia, as noted in the case of hereditary nonpolyposis colon cancer (HNPCC) in humans (Atkin, 2001, Adkinson and Brown, 2007). MI has also been seen in other cancers such as endometrial carcinoma and gastric adenocarcinoma. However in these cancers an association with mutations in the *MMR* is less obvious (Gurin et al., 1999, Atkin, 2001) and it is noted that other genes involved with maintaining genetic integrity are still unknown (Sieber et

al., 2005, Hussein and Wood, 2002). MI has been used as a prognostic marker for disease as it has been noted that tumours that are microsatellite stable offer a better prognosis than those which show even a low level of instability (Hussein and Wood, 2002).

Genes implicated in the development of neoplasia can be broadly split into two categories; oncogenes and tumour suppressor genes as reviewed by Adkinson and Brown, 2007,Chow, 2010 and Bunz, 2008 (Adkinson and Brown, 2007, Chow, 2010, Bunz, 2008). Oncogenes are genes that due to a mutation have acquired the ability to enhance cell proliferation in the absence of appropriate signals, due to the production of an erroneous protein (Adkinson and Brown, 2007, Bunz, 2008, Chow, 2010). Additionally, oncogene acquisition may occur in animals via viral infection with or without integration of genetic material, for example in the non-acute and acute transforming retroviruses respectively (Maeda et al., 2008). Oncogenes act in a dominant fashion therefore only one affected allele is necessary to produce an oncogenic effect (Adkinson and Brown, 2007). In general oncogenes have their origins in proto-oncogenes; these are normally functioning genes that are involved in the process of cell division (Bunz, 2008, Chow, 2010).

As their name suggests, tumour suppressor genes act in an opposite way to oncogenes; they function to suppress the proliferation of cells, however if they are mutated they can lose this action (Adkinson and Brown, 2007, Bunz, 2008). In comparison to the dominant way in which oncogenes operate, mutated tumour suppressor genes lose the function of both alleles and therefore assist the development of neoplasia in a recessive manner (Adkinson and Brown, 2007, Chow, 2010). One of the most important tumour suppressor genes is Tumour Protein 53 (*TP53*) which codes for the protein p53. This protein is expressed when the cell is affected by factors that cause mutations, it acts to halt cell division until the cell has repaired the damage (Meek, 2009, Harris, 1996). Both *TP53* alleles are required to be inactivated for neoplasia to occur. It is this gene that features in familial Li-Fraumeni syndrome, where the gene is transmitted in an autosomal dominant manner (Bunz, 2008, Pantziarka, 2013, Li et al., 1988). Members of a family carrying this gene therefore only require the mutation of one *TP53* allele for disease to occur. A variety of neoplastic conditions are reported to occur at a young age

in members of families carrying this gene (Pantziarka, 2013, Adkinson and Brown, 2007, Malkin, 1994).

It has been suggested that tumour suppressor genes may occur in regions of the genome affected by LOH (Wang et al., 2004). It has been identified that particular regions of the genome are predisposed to LOH which can increase the risk of neoplasia, including urogenital carcinoma in humans (Cheung et al., 2005, McKenzie et al., 2000, Peiffer et al., 1995, Smith et al., 1992). In CSL the location of *HPSE2* in the genome has not been determined, however in the human genome it is located at 10q23-24 (McKenzie et al., 2000). This is noted as being in a so-called loss of heterozygosity LOH region (McKenzie et al., 2000, Thiagalingam et al., 2002). Thaigalingam et al., (2002) summarises seven potential ways LOH may arise; localized deletion, gene conversion, mitotic recombination, translocation, chromosome breakage and loss, chromosome loss or chromosome loss and duplication. In cases where one of the alleles has already undergone a mutation, as has been noted in familial retinoblastoma, the potential loss of the unaffected allele leaves the individual at risk of developing neoplasia (Thiagalingam et al., 2002). Although at this stage it is unclear whether *HPSE2* is a tumour suppressor gene or an oncogene, it is interesting that in humans it is located close to the tumour suppressor gene phosphatise and tensin homolog (*PTEN*) at 10q23-25 (Thiagalingam et al., 2002, McKenzie et al., 2000, Li et al., 1997). *PTEN* is associated with a number of cancers including genital, (Bunz, 2008, Ali et al., 1999, Li et al., 1997). The *PTEN* gene is stated as being "one of the most commonly mutated tumour suppressors in human cancer" (Salmena et al., 2008).

Due to the identification of the Pv11 microsatellite within the *HPSE2* gene and its association with UGC in the CSL, the study planned to investigate whether genetic instability was present at this locus. Initially the study aimed to (i) identify the structure of the Pv11 microsatellite in the five allele types identified, (ii) using DNA extracted from skin and DNA extracted from corresponding urogenital tract tissues from the same animal, compare sequences of the Pv11 microsatellite of each allele type. Comparing DNA from the two tissues would establish if there was any indication of MI at the Pv11 locus that could potentially affect RNA splicing and translation of the *HPSE2* gene and (iii) to investigate whether LOH was occurring by comparing electropherograms of

PCRs of the Pv11 locus from DNA extracted from skin and lower genital tract tissue from the same animal. Identification of LOH would assist in ascertaining the putative function of *HPSE2*.

4.2 Materials and Methods

4.2.1 Investigation of Pv11 structure

Sample selection

Where possible, animals previously identified as homozygous in Chapter 2 at each of the five alleles were selected. In total 32 homozygotes were identified consisting of nineteen allele one, six allele two, five allele three and two allele four animals, due to an absence of allele five homozygotes, two heterozygotes were included. Corresponding lower genital tract tissue was available for 30 of the animals.

DNA extraction and preparation

DNA was extracted from skin samples as previously described in Chapter 2. DNA from the lower genital tract tissue was extracted with the inclusion of an additional incubation step with alpha amylase (Sigma-Aldrich Ltd, Gillingham, UK) 10% by volume for 2 h at 37°C, prior to the addition of RNase A. This was as per the protocol used by Buckles et al., (2006) in order to remove tissue proteoglycans. Skin and lower genital tract DNA was quantified as described in Chapter 2 and diluted with MilliQ water to working stocks of 10 ng/µl and stored at -20°C. To prevent confusion with DNA extracted from the skin, the samples extracted from the lower genital tract were given an additional number (shown in brackets after the accession number).

Amplification of Pv11

A nested PCR protocol was employed in order to increase the specificity of the PCR to amplify a 719 bp of sequence in intron 9 that included the Pv11 microsatellite for the purpose of sequencing. The reaction mixture for both the primary and nested PCR consisted of 25 µl reactions of 2.5 µl of *Pfu* DNA polymerase 10 X buffer (Promega, Southampton, UK), 0.5 µl 10 mM dNTPs (Qiagen, Crawley, UK), 0.5µl of forward and

reverse primer from 50 µM stock solutions, 0.21 µl *Pfu* DNA polymerase (0.63 units) (Promega, Southampton, UK), 19.79 µl RNase free water (Qiagen, Crawley, UK) and 1 µl of 10 ng/µl DNA template in the case of the primary reaction. For the nested reaction the product of the primary reaction was diluted 1:5 with RNase free water (Qiagen, Crawley, UK) and 1 µl of this was used as the template. The primers used were designed based on preliminary sequences obtained from CSL of the intron containing Pv11, in addition sequences from the following species were examined; canine, equine, bovine and harbour seal (*Phoca vitulina*). This enabled the identification of conserved regions for the design of primer sites, short regions of the alignment along with the primer sites are illustrated in Figure 4.1. The primers were obtained from Invitrogen (Paisley, UK) and the sequences are detailed in Table 4.1.

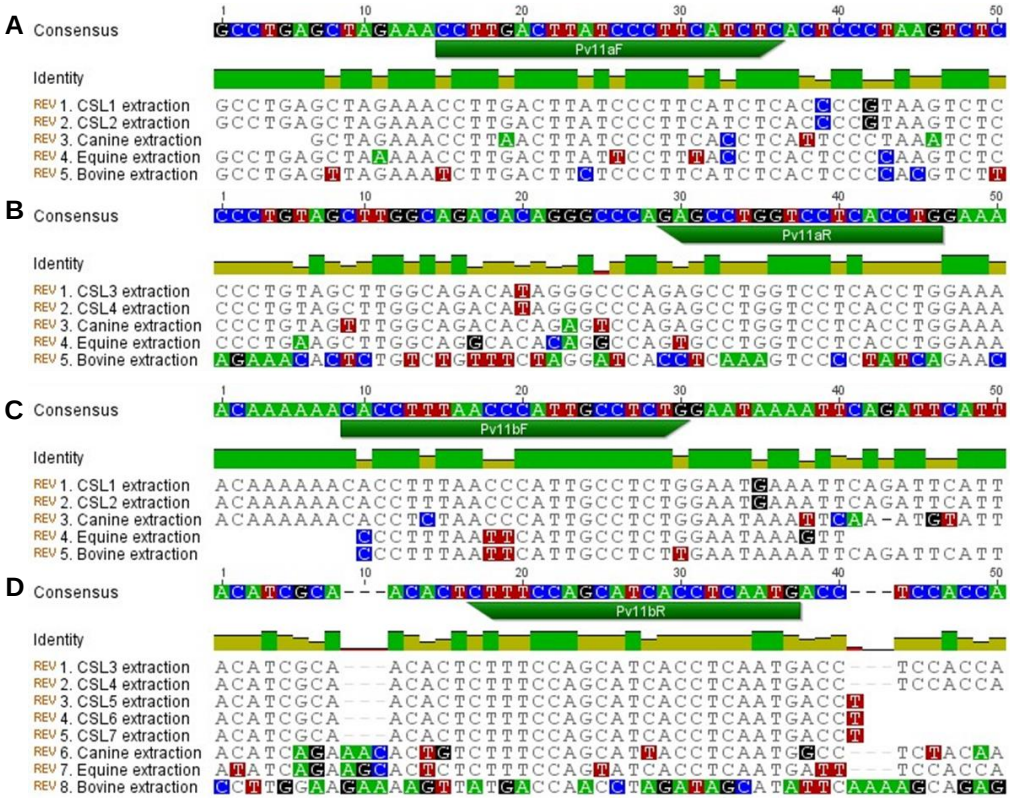


Fig 4.1 The primers used in the PCR to amplify a fragment across the Pv11 microsatellite in order to sequence it were designed from an alignment of the following species; CSL, canine, equine and bovine. Short regions of the alignment are illustrated above to show how the primers were designed. A and B show the regions used to design the forward and reverse primers used in the primary PCR; C and D show the regions used to design the forward and reverse primers used in the secondary PCR.

Table 4.1 Primer sequences used in the nested PCR for sequencing the Pv11 locus

Primer	Sequence 5'-3'	Reference
Pv11a	F: CCT TGA CTT ATC CCT TCA TCT C	(This study)
(primary)	R: CAG GTG AGG ACC AGG CTC	
Pv11b	F: CAC CTT TAA CCC ATT GCC TCT G	(This study)
(nested)	R: CAT TGA GGT GAT GCT GGA AAG	

A negative control was included in both the primary and nested reactions by substituting DNA template with the same volume of autoclaved MilliQ water. Amplification of the microsatellite marker was carried out in a PTC-200 DNA Engine Cycler (Bio-Rad Laboratories Inc., Hercules, USA) using the following temperature cycling conditions for both primary and nested reactions; 95°C for 2 min, followed by 35 cycles of 95°C for 45 s, 50°C for 30 s, 72°C for 4 min, followed by a final extension step of 72°C for 5 min before being stored at 4°C.

Fragment analysis

To analyse the products, 5 µl of PCR product was combined with 2.5 µl Orange G loading dye (Sigma-Aldrich Ltd, Gillingham, UK) and resolved at 80 V for 30 min on a 1.5% agarose gel containing 2.5 µl ethidium bromide (Sigma-Aldrich Ltd, Gillingham, UK) alongside a 1 kb ladder (Invitrogen, Paisley, UK). Bands were visualised in a UV light box (UVITEC, Cambridge, UK). Samples showing successful amplification on gel electrophoresis were purified using MSB® Spin PCRapace PCR purification kits (Stratec molecular, Berlin, Germany) and then quantified by running 5 µl of the purified product alongside three lambda DNA size markers: 25 ng, 50 ng and 100 ng (Promega, Southampton, UK) on a 1% agarose gel. Where possible, 40 ng of PCR product was submitted for sequencing along with 3.2 pmol of both forward (Pv11bF) and reverse (Pv11bR) primer from the nested reaction.

Sequencing

DNA sequencing was performed by DNA Sequencing & Services (MRCPPU, College of Life Sciences, University of Dundee, Scotland, www.dnaseq.co.uk) using Applied Biosystems Big-Dye Ver 3.1 chemistry on an Applied Biosystems model 3730

automated capillary sequencer. The DNA sequences were analysed to identify where variations occurred within the repeat unit of the microsatellite and in addition to compare sequences obtained from amplification of the region using skin DNA as opposed to DNA extracted from lower genital tract tissue. DNA sequence analysis was performed using the software programme Geneious Pro v5.6.6 created by Biomatters. Available from http://www.geneious.com/).

4.2.2 Loss of Heterozygosity

Sample selection

To investigate the occurrence of LOH, the Pv11 microsatellite marker was amplified alone within skin and corresponding lower genital tract tissue in 128 samples (64 skin DNA samples and 64 corresponding lower genital tract DNA samples) from 34 previously genotyped heterozygote animals and 30 homozygote animals. Homozygotes were included in the experiment as controls to confirm consistency in amplification of Pv11 due to previous knowledge of their genotype. The heterozygotes were made up of seven cases and 27 controls.

DNA extraction and preparation

DNA was extracted from skin and lower genital tract tissue as previously.

Amplification of Pv11

The PCR undertaken was similar to that described in Chapter 2 with the modification of only one microsatellite being amplified. The reactions consisted of 10 µl mixtures of 20 ng DNA template from either skin or lower genital tract, 5 µl 2×Qiagen Multiplex Master Mix (Qiagen, Crawley, UK) and 3 µl of primer mix. The primer mix was prepared by combining 6 pmol of forward and reverse primer along with 3.3 pmol of Pv11 forward primer with a D3 fluorescent tag (green) and 2.7 µl RNase-free water (Qiagen, Crawley, UK). Primers were obtained from Invitrogen (Paisley, UK) and fluorescent tags from Sigma-Aldrich Ltd (Gillingham, UK). Primer sequences for Pv11 amplification are detailed in Table 2.1 in Chapter 2. Amplification of Pv11 was carried out in a PTC-200 DNA Engine Cycler (Bio-Rad Laboratories Inc., Hercules, USA), using the following temperature cycling conditions: 95°C for 15 min, followed by 35

cycles of 94°C for 30 s, 57°C for 90 s, 72°C for 45 s, followed by a final extension step of 72 °C for 10 min. The samples were stored at 4°C prior to analysis.

Fragment analysis

Fragment analysis was achieved via automated capillary electrophoresis followed by peak analysis using CEQ specific software as described in Chapter 2. In addition to ascertaining whether samples where homo or heterozygous, the peak heights of the fluorescent signal peaks were recorded to allow comparison of the signal strength between samples from skin DNA and those from lower genital tract DNA to enable LOH calculations. To check for errors in the amplification 30% of the samples were run twice and two negative controls per 96 well plate were included to highlight any contamination should it occur.

LOH analysis

LOH was investigated using the formula $(N_2/N_1)/(T_2/T_1)$ where N is the peak height of the assumed normal alleles (in this case in the samples of DNA from the skin) and T is the peak height of the potential abnormal alleles (in this case the samples of DNA from the lower genital tract tissue). Using this formula, LOH is strongly suggested if the ratio is <0.5 or >2.0 (Poetsch et al., 2004, Dietmaier et al., 1997).

4.2.3. Statistical analysis

In the event of the identification of LOH, a Fisher's exact test was carried out to establish significance using the open access statistical software package (R Development Core Team, 2012).

4.2.4 Problems encountered

Similarly to the genotyping in Chapter 2, contamination was an issue with peaks appearing in the water negative control wells on analysis, this was remedied by UV irradiation as in Chapter 2 and with purchasing a new capillary array. The removal of a heterozygote from the study was required due to the erroneous sampling of a homozygote of similar animal number; 8921(33), a homozygote with a 4,4 Pv11 genotype was confused with 9821(72) a heterozygote with a 1,2 Pv11 genotype.

Although consistency in amplification was noted by comparison of the allele profile recorded from the multiplex PCR reactions undertaken in Chapter 2, the actual size of the five Pv11 alleles identified here were five base pairs larger. The reaction mixtures, primers and analysis method were the same with the exception that only the Pv11 microsatellite, rather than a multiplex reaction of the three microsatellites was undertaken in this experiment. The nested sequencing PCR traces were analysed using the primer sequences used for genotyping in Chapters 2 and 4 (Table 2.1) and it was found that the actual size of Pv11 was different again (Table 4.2). This should be remembered if the actual fragment size is required.

Table 4.2 Variation in Pv11 allele size identified in different experiments

Allele	Multiplex PCR (bp)	Pv11 only (bp)	Actual (bp)*
1	176	181	**179**
2	178	183	**181**
3	180	185	**183**
4	182	187	**185**
5	184	189	**X**

* Including primer sequences; X: Insufficient sequence quality to identify both primer sites; bp: base pairs

4.3 Results

4.3.1 Pv11 structure and microsatellite instability

Due to the previously identified association of homozygosity at the Pv11 locus with UGC (Chapter 2) the structure of different Pv11 alleles in normal unaffected tissue (skin) was investigated in comparison to that in genital tissue in order to establish both normal structure and identify possible instability. The Pv11 microsatellite was amplified successfully in the majority of samples from both skin DNA and DNA extracted from lower genital tract tissue. Six were deemed of inadequate quality for analysis (Table 4.3). An example gel electrophoresis of PCR products is illustrated in Figure 4.2. Subsequent sequencing of the purified products resulted in approximately 200-300 bp of good quality sequence from both the forward and reverse reads (except in the case of the heterozygotes including allele five where less good quality sequence was obtained) and included the Pv11 microsatellite. This allowed both the structure of the microsatellite to be gained along with sequence comparison of the variable microsatellite region within individual animals from both healthy and diseased tissue of different Pv11 genotypes. The position of the primers used in the PCR in relation to the structure of the *HPSE2* gene is indicated in Figure 4.3.

The structure of the five Pv11 alleles was almost identical in DNA from the skin and the genital tract from the same individuals. The microsatellites were polymorphic and comprised a variable region of CA dinucleotide units that were preceded by seven AC dinucleotide units (Figure 4.4). The end of the microsatellite had a mononucleotide C repeat sequence that also varied in number depending on the allele (Table 4.3). The structure of the alleles was as follows; allele one: (AC)7G(CA)13(C)4, allele two: (AC)7G(CA)13(C)6, allele three: (AC)7G(CA)15(C)4, allele four: (AC)7G(CA)16(C)4 and allele five: (AC)7G(CA)17(C)3. Allelic imbalance in the form of an apparent microsatellite contraction in the CA repeat was observed in three animals; two allele two control animals and one allele two UGC animal all showing (CA)13→(CA)12 difference between skin and genital tract DNA (Table 4.3). Conclusions from such limited instability cannot be made, especially considering potential *taq* polymerase slippage during PCR. In all animals the preceding AC repeat units and the mononucleotide C repeat sequences were unaltered between the tissues (Table 4.3).

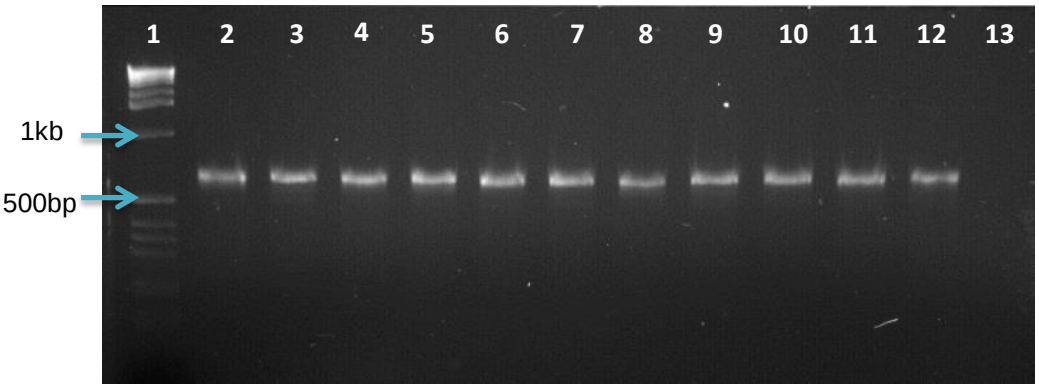


Fig 4.2 Gel electrophoresis of 5 µl of nested Pv11 PCR product of lower genital tract DNA to confirm presence of appropriate sized fragment. Lane 1] 1kb ladder, 2] 7972(74), 3] 7997(68), 4] 8431(69), 5] (7867(73), 6] 9339(70), 7] (9572(71), 8] (9325(76), 9] (7819(75), 10] (9821(72), 11] (9184(14), 12] (9724(25), 13] Negative control.

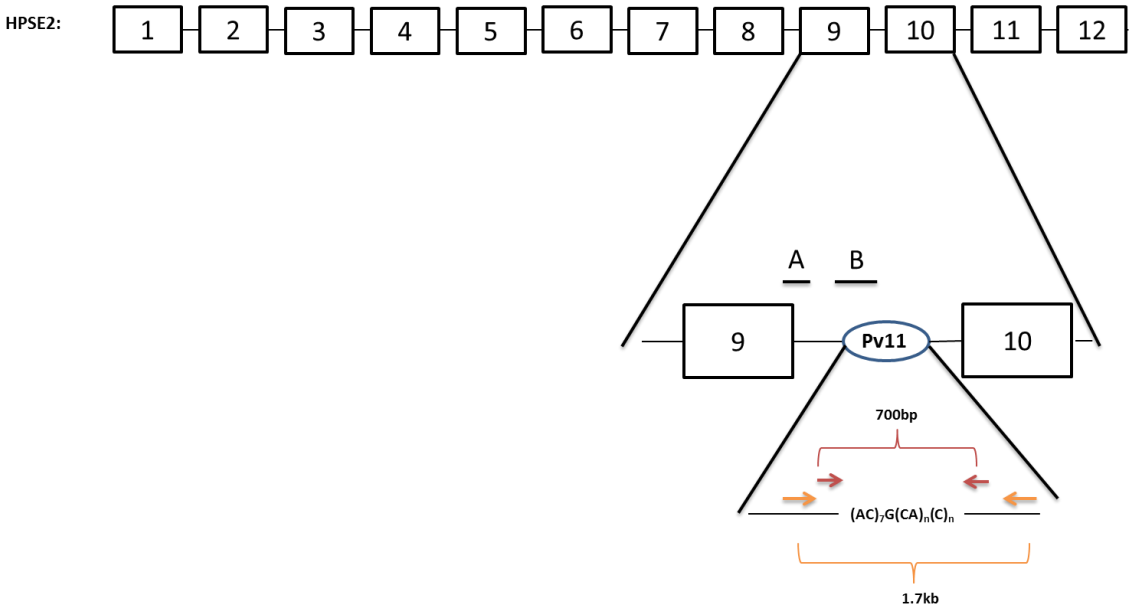


Fig 4.3: Structure of the *HPSE2* gene is conserved amongst mammals and consists of 12 exons separated by large intronic regions. The first pop out window illustrates the position of the two probes (A and B) employed in the southern blot. Probe A being ~500bp in size and incorporating 22bp of exon 9 and probe B being ~1kb in size and flanking the Pv11 microsatellite. The second pop out window illustrates the position of the two sets of primers used to amplify the Pv11 region prior to sequencing the microsatellite; the light orange arrows indicate primers used to amplify the ~1.7kb fragment in the primary PCR and the red arrows indicate the primers used in the nested reaction to amplify the ~700bp fragment which was submitted for sequencing.

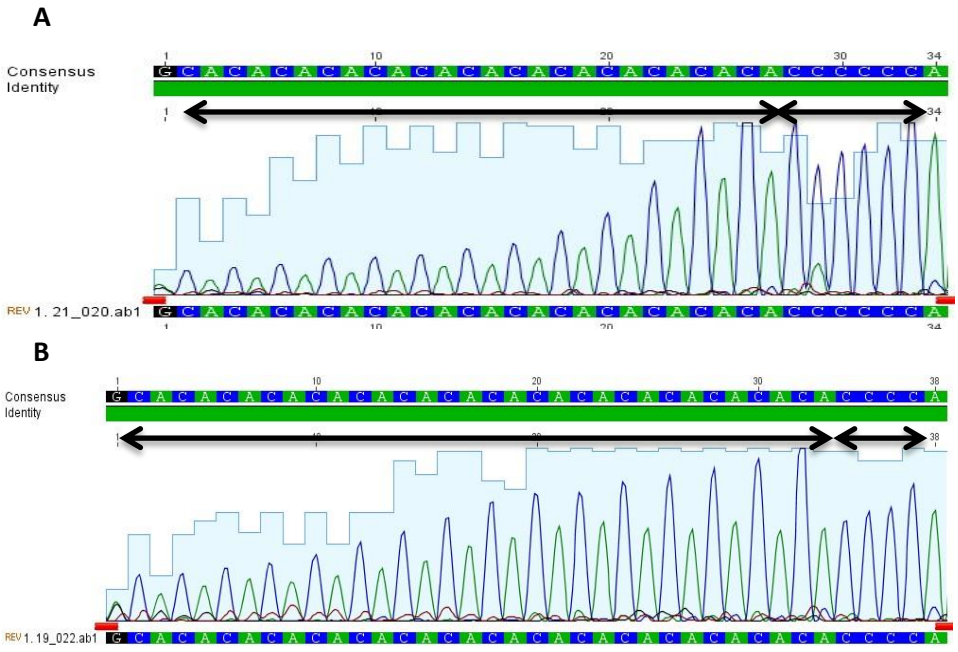


Fig 4.4 Electropherogram of reverse primer sequencing results revealing the Pv11 variable dinucleotide (CA) and mononucleotide (C) regions in DNA from skin, indicated by the black arrows. Height of light blue background indicates confidence that the base is the one that is shown (higher the level of light blue the more confident). A: Animal 7750, an allele two showing (CA)13 and (C)6; B: Animal 8921(33) an allele four showing (CA)16 and (C)4.

Table 4.3. Pv11 sequencing results including those showing apparent allelic imbalance (indicated in red). Allele five sequence was identified by excluding the presence of the allele two sequence.

Animal No.	Pv11 genotype	UGC positive/Control	Skin DNA	Genital tissue DNA
9184(14)	1,1	Control	$(AC)_7G(CA)_{13}(C)_4$	$(AC)_7G(CA)_{13}(C)_4$
9325(76)	1,1	Control	$(AC)_7G(CA)_{13}(C)_4$	$(AC)_7G(CA)_{13}(C)_4$
9114(2)	1,1	Control	$(AC)_7G(CA)_{13}(C)_4$	$(AC)_7G(CA)_{13}(C)_4$
9303(36)	1,1	Control	X	$(AC)_7G(CA)_{13}(C)_4$
9463(28)	1,1	Control	$(AC)_7G(CA)_{13}(C)_4$	X
9100(77)	1,1	Control	$(AC)_7G(CA)_{13}(C)_4$	$(AC)_7G(CA)_{13}(C)_4$
9196(78)	1,1	Control	$(AC)_7G(CA)_{13}(C)_4$	$(AC)_7G(CA)_{13}(C)_4$
8958(79)	1,1	Control	$(AC)_7G(CA)_{13}(C)_4$	$(AC)_7G(CA)_{13}(C)_4$
9008(80)	1,1	Control	$(AC)_7G(CA)_{13}(C)_4$	$(AC)_7G(CA)_{13}(C)_4$
9804(81)	1,1	UGC positive	$(AC)_7G(CA)_{13}(C)_4$	$(AC)_7G(CA)_{13}(C)_4$
7972(74)	1,1	UGC positive	$(AC)_7G(CA)_{13}(C)_4$	$(AC)_7G(CA)_{13}(C)_4$
7997(68)	1,1	UGC positive	$(AC)_7G(CA)_{13}(C)_4$	$(AC)_7G(CA)_{13}(C)_4$
8431(69)	1,1	UGC positive	$(AC)_7G(CA)_{13}(C)_4$	$(AC)_7G(CA)_{13}(C)_4$
9757(39)	1,1	UGC positive	$(AC)_7G(CA)_{13}(C)_4$	$(AC)_7G(CA)_{13}(C)_4$
9827(41)	1,1	UGC positive	$(AC)_7G(CA)_{13}(C)_4$	$(AC)_7G(CA)_{13}(C)_4$
9911(34)	1,1	UGC positive	$(AC)_7G(CA)_{13}(C)_4$	$(AC)_7G(CA)_{13}(C)_4$
7140(82)	1,1	UGC positive	$(AC)_7G(CA)_{13}(C)_4$	$(AC)_7G(CA)_{13}(C)_4$
7468(83)	1,1	UGC positive	$(AC)_7G(CA)_{13}(C)_4$	$(AC)_7G(CA)_{13}(C)_4$
6370	1,1	UGC positive	$(AC)_7G(CA)_{13}(C)_4$	XX
7819(75)	2,2	Control	$(AC)_7G(CA)_{13}(C)_6$	$(AC)_7G(CA)_{13}(C)_6$
9597(43)	2,2	Control	$(AC)_7G(CA)_{13}(C)_6$	$(AC)_7G\mathbf{(CA)_{12}}(C)_6$
7750	2,2	Control	$(AC)_7G(CA)_{13}(C)_6$	XX
8029(84)	2,2	Control	$(AC)_7G(CA)_{13}(C)_6$	$(AC)_7G\mathbf{(CA)_{12}}(C)_6$
9724(25)	2,2	UGC positive	$(AC)_7G(CA)_{13}(C)_6$	$(AC)_7G(CA)_{13}(C)_6$
9770(42)	2,2	UGC positive	$(AC)_7G(CA)_{13}(C)_6$	$(AC)_7G\mathbf{(CA)_{12}}(C)_6$
7131	3,3	Control	$(AC)_7G(CA)_{15}(C)_4$	XX
7867(73)	3,3	UGC positive	$(AC)_7G(CA)_{15}(C)_4$	$(AC)_7G(CA)_{15}(C)_4$
9339(70)	3,3	UGC positive	$(AC)_7G(CA)_{15}(C)_4$	$(AC)_7G(CA)_{15}(C)_4$
9572(71)	3,3	UGC positive	$(AC)_7G(CA)_{15}(C)_4$	$(AC)_7G(CA)_{15}(C)_4$
8059(30)	3,3	UGC positive	$(AC)_7G(CA)_{15}(C)_4$	$(AC)_7G(CA)_{15}(C)_4$
7159	4,4	Control	$(AC)_7G(CA)_{16}(C)_4$	XX
8921(33)	4,4	UGC positive	$(AC)_7G(CA)_{16}(C)_4$	X
9254(3)	2,5	Control	$(AC)_7G(CA)_{17}(C)_3$	X
9871(23)	3,5	Control	X	X

XX: Sequence unavailable due to absence of tissue sample; X: inadequate sample quality

4.3.2 Loss of Heterozygosity

Particular regions of the genome are predisposed to a loss of heterozygosity (LOH) which can increase the risk of neoplasia, including urogenital carcinoma in humans (Cheung et al., 2005, McKenzie et al., 2000, Smith et al., 1992). LOH at Pv11 was investigated by PCR using DNA from skin and lower genital tract tissues, utilising fluorescently tagged primers targeted to the Pv11 microsatellite followed by detection by capillary electrophoresis. This technique allowed analysis of the intensity of the fluorescent signal to identify if LOH, either partial or full, was occurring (Dietmaier et al., 1999, Poetsch et al., 2004).

Consistency of Pv11 amplification was noted in the samples by comparison with the genotyping results in Chapter 2 and of the 128 samples two lower genital tract samples (both from homozygotes) failed to amplify. In all of the heterozygotes in the study amplification of Pv11 in both tissues was successful. However one heterozygote was removed from the study due to the sampling error described previously. Analysis of the 33 remaining Pv11 heterozygote animals (consisting of 26 control animals and seven diagnosed with UGC), identified LOH in one UGC animal (Figure 4.5). Animal 9904(45) had an LOH ratio of 2.27, with ratios of <0.5 or >2 being strongly suggestive of LOH (Dietmaier et al., 1999, Poetsch et al., 2004). However, the Pv11 genotype of this animal remained as 1,3, suggesting partial allele loss rather than complete LOH. The spread of the LOH ratios calculated from the control animals ranged from 0.73 – 1.23 and the spread of ratios from the UGC animals ranged from 0.90 – 2.27 (Figure 4.6).

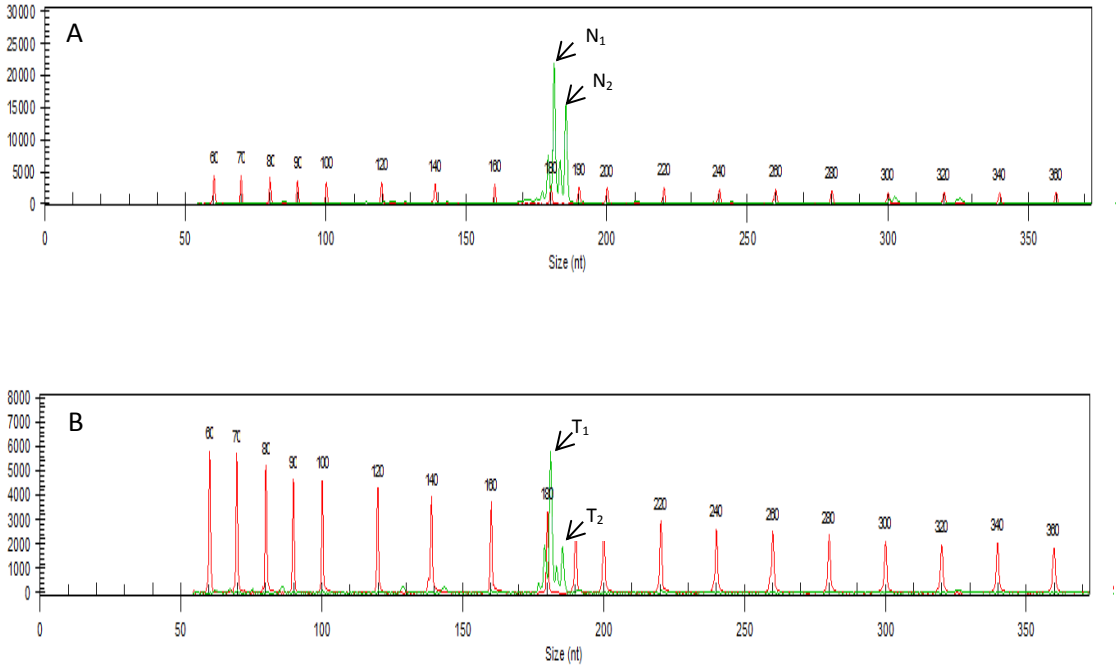


Fig 4.5. Electropherograms of microsatellite Pv11 PCR in DNA from skin (A) and urogenital tract (B). Red peaks are 400 bp size standard and green peaks are Pv11 microsatellite alleles of sample 9904(45). N_1 and N_2 are alleles amplified from presumed normal tissue, T_1 and T_2 are alleles amplified from presumed tumour tissue. LOH ratio of peak height $(N_2/N_1)/(T_2/T_1)$ was 2.27 suggesting partial allele loss.

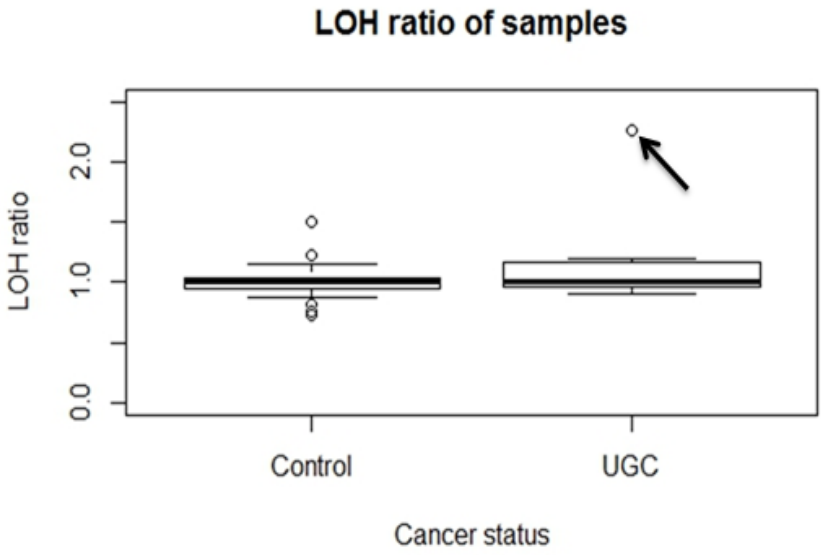


Fig 4.6. Boxplot illustrating the range of LOH ratios, the majority being in the normal range of >0.5 and <2 except for animal number 9904(45) which had a ratio of 2.27 indicated by the arrow.

Statistical analysis

The results of the fishers exact test indicated that LOH is not significantly associated with the occurrence of UGC in the CSLs examined in this study (p=0.212).

4.4 Discussion

Alterations in the length of a microsatellite can affect protein coding of a gene and disease susceptibility as in the example discussed in Chapter 2 concerning the CA repeat in intron one of the epidermal growth factor receptor gene (*EGFR*) (Vashist et al., 2013, Suzuki et al., 2008). The structure of Pv11 in the present study revealed an increasing CA repeat unit with increasing allele number, yet statistical analyses undertaken (Chapter 2) didn't find any evidence that a particular allele was significantly associated with UGC. However, this may be a result of the small sample size. Out of the 25 animals examined where successful amplification of Pv11 in both skin and lower genital tract tissue was achieved, apparent MI was seen in the form of microsatellite contraction in three allele two animals, consisting of two controls and one UGC positive. Although determining whether this is due to *Taq* polymerase slippage during PCR or is a result of true instability is not possible (Clarke et al., 2001), however if instability is occurring in the microsatellite it is rare.

Pv11 was the only microsatellite examined for instability in the present study. In studies involving MI in human colorectal cancer a panel of five microsatellites known to be prone to instability are used and the extent of instability graded according to the number of microsatellites exhibiting it (Boland et al., 1998, Dietmaier et al., 1997, Dietmaier et al., 1999). It is unknown whether defects in the *MMR* are a contributing factor to UGC in CSL and additionally the susceptibility of Pv11 to MI is unknown. This along with the small sample size and the lack of micro-dissection carried out on the lower genital tract tissues prior to DNA extraction may contribute to the absence of clearly identifiable MI. Micro-dissection is necessary to be certain that DNA is extracted from tumour affected cells rather than normal tissue (Boland et al., 1998, Nishimura et al., 2000, Dietmaier et al., 1999).

The lack of micro-dissection may also have contributed to only a single case of LOH being identified, therefore in spite of the non-statistically significant finding further investigation is warranted as it is possible that other cases were not detected because of the experimental methods used. In future investigations cases should be subjected to more meticulous sample taking and preparation, additionally the analysis of a larger number of animals would be beneficial. The location of *HPSE2* in the human genome (10q23-24) reinforces the need for more studies as it is noted to be in an LOH region, regions which are believed to contain tumour suppressor genes (Wang et al., 2004). Further to this expression of the *HPSE2* gene is repressed by the Polycomb Group (PcG) protein (*EZH2)* and genes supressed by this are thought to be tumour suppressors (Yu et al., 2007, Levy-Adam et al., 2010). *EZH2* itself is considered a marker of invasive breast cancer (Kleer et al., 2003, Yu et al., 2007). These findings support the idea that *HPSE2* is potentially a tumour suppressor rather than an oncogene, however information regarding the activity of this gene in cancer is still scarce and although increased expression of its protein has been recognised in a few cancer studies (Table 3.2) its true nature is presently unknown (Levy-Adam et al., 2010). LOH of the 10q23-24 region in humans has been identified in a number of cancers including hepatocellular carcinoma, cervical carcinoma, prostate cancer, small cell lung cancer and medulloblastoma (Okuno et al., 2009, Rizvi et al., 2012, Leube et al., 2002, Kim et al., 1998, Scott et al., 2006). The tumour suppressor gene *PTEN* is also found in this chromosomal region and in many cases it is suggested as a candidate gene, however studies also acknowledge that there may be other unknown genes in the vicinity that are involved in the neoplasm in question (Kim et al., 1998, Leube et al., 2002, Okuno et al., 2009, Scott et al., 2006).

Stage of disease may also be a factor in whether MI or LOH is detected. In breast cancer it was found that MI occurred early on in the course of disease whereas LOH appeared to occur later (Sourvinos et al., 1997) this is in comparison to uterine cervical cancer where the opposite was reported (Nishimura et al., 2000). It is therefore possible that time of sampling within the course of the disease in CSL may contribute to whether genetic instability is detected or not. Although course of disease in the CSL is unknown, the necropsy histopathology reports of the animals entered into the study suggest many of them were in an advanced state of disease.

In this study genetic instability in the form of MI and LOH was not found to be significantly associated with the presence of cancer, however as discussed more work is required to confirm that instability is not part of the molecular pathogenesis of this disease.

Chapter 5

Characterisation of the activity of the Heparanase 2 (*HPSE2*) gene in urogenital tract tissue from the California sea lion (*Zalophus californianus*) and its association with the presence of urogenital carcinoma.

5.1 Introduction

5.1.1 Basics of gene expression

The process of gene expression occurs in two main stages; transcription occurring in the nucleus of the cell and translation in the cytoplasm (Clancy, 2008a, Phillips, 2008, Alberts et al., 1994). Transcription describes the replication of DNA into an RNA copy and comprises three steps starting with initiation. In initiation RNA polymerase binds to the DNA helix at the promoter sequence, this is followed by the elongation step where the DNA helix is opened exposing the template strand and allowing the subsequent addition of nucleotides at the 3 prime (3') end. Nucleotides are made up of three components, a base, a phosphate molecule and a ribose sugar. The 3' end refers to the ribose end of the molecule, conversely the end containing the phosphate molecule is called the 5 prime (5') end (Nicholas, 2010a). The final step of transcription is termination and when the RNA polymerase reaches a terminator sequence it results in the release of the RNA polymerase and new RNA transcript (Clancy, 2008a). At this stage the RNA transcript is called pre-messenger RNA (pre-mRNA), until it undergoes post transcriptional modification by 5' capping with a methylated guanine nucleotide, polyadenylation and splicing into messenger RNA (mRNA) (Minvielle-Sebastia and Keller, 1999, Smith and Valcarcel, 2000) . The mRNA transcript then moves out of the nucleus and into the cytoplasm where translation begins (Alberts et al., 1994, Avison, 2007).

During translation, ribosomal subunits in the cytoplasm consisting of a large and a small subunit join onto the mRNA transcript and move in a 5' to 3' direction along the mRNA. Transfer RNA (tRNA) carrying amino acids corresponding to the triplet code on the mRNA are placed in sequence with the formation of peptide bonds between them

resulting in a polypeptide chain. Translation always starts with a codon corresponding to the methionine (MET) amino acid and ends with one of three stop codons. When a stop codon is reached translation is ceased and the polypeptide chain along with the mRNA is released from the ribosomes (Huang et al., 2011, Avison, 2007, Clancy and Brown, 2008). Post translational modification of the polypeptide then occurs with folding of the polypeptide chain. The resultant shape is dependent on the different affinities of the amino acids present and the action of additional protein molecules called chaperones (Vabulas et al., 2010).

All cells contain the same genes however which ones are expressed varies between the tissues, this is known as differential expression and explains the different phenotypes of tissues (Smith, 1990, Evans and Wheeler, 2001). The exceptions are the so called housekeeping genes which are required for all cellular functions and are believed to be expressed at a relatively constant level. However, even expression of these varies to some extent and care is required in choosing particular housekeeping genes as controls in expression studies (Silver et al., 2006, Eisenberg and Levanon, 2013). When gene transcription occurs it is estimated that only up to 40% of mRNA is translated into protein, therefore identifying the presence of mRNA does not necessary mean that the protein is present (Huang et al., 2011, Nie et al., 2006, Tian et al., 2004). Control of gene expression to the subsequent production of an active protein can occur at various stages from transcriptional control by transcription factors (Phillips and Hoopes, 2008, Yang, 1998) to the control of translation of mRNA transcripts via mRNA silencing by microRNAs (miRNA) and short interfering RNAs (siRNA) (Valencia-Sanchez et al., 2006, Wu et al., 2006).

The number of proteins produced by the human genome exceeds the number of genes present; this is possible due to a post transcriptional modification called alternative splicing (As) (Faustino and Cooper, 2003, Dutertre et al., 2011, Smith and Valcarcel, 2000). Splicing removes the introns from the mRNA prior to translation, whereas As removes both introns along with some exons allowing the expression of one gene to result in more than one protein product or isoform (Berget, 1995, Clancy, 2008b). Splicing and As take place in the nucleus of the cell by the action of a large ribonucleoprotein structure called the spliceosome (Will and Luhrmann, 2011). As has

been noted to occur in *HPSE2* expression in humans where four isoforms have been reported (Daly et al., 2010), similarly three isoforms have been detected in the domestic dog (NCBI UniGene: XM_856105).

5.1.2 Gene expression studies and disease

Gene expression studies are frequently undertaken in disease investigations and comparisons of expression (of both mRNA and protein) between affected and unaffected individuals can assist in investigating the postulated involvement of a gene or molecular pathway. Over the past 20 years gene expression studies have advanced to include the use of microarrays including in CSLs (see Chapter 7). Microarrays enable the examination of the up or down regulation of a number of pre-selected genes at once (Trevino et al., 2007, Schena et al., 1995). Examples of gene expression studies in veterinary species include the identification of differing expression patterns in canine atopic dermatitis and canine brain tumours (Merryman-Simpson et al., 2008, Thomson et al., 2005). These studies led to the identification of candidate genes for future investigation and in the case of canine brain tumours the discovery of genetic similarities with the condition in humans. In addition to assessment of differences between the activity of genes in affected and unaffected tissues, identification of the protein is useful both as a prognostic and diagnostic tool (Weinstein et al., 2002, Lahoti et al., 1996). In the case of HPA2, discussed in Chapter 3, the presence of the protein has been identified as a prognostic marker in both gastric and head and neck carcinoma (Levy-Adam et al., 2010, Zhang et al., 2013).

Homozygosity at the Pv11 locus was found to be significantly associated with UGC. Further investigation failed to identify any significant finding concerning Pv11 structure (Chapter 4), however due to the location of Pv11 within *HPSE2*; expression of this large gene was examined. The present study aimed to fulfil a number of objectives; (i) to establish if *HPSE2* is expressed within tissues of the lower genital tract in female CSL, namely in vaginal and cervix tissue and (ii) to identify the presence or absence of isoforms and to compare the expression profile of different genotypes of both UGC positive and control animals and (iii) to investigate the presence of the expressed protein

in the lower genital tract of animals of different genotypes. Both UGC positive and control animals will be investigated, with UGC positive cases consisting of samples from animals of differing histological disease grade.

5.2 Materials and Methods

5.2.1 Investigating *HPSE2* Transcription

Sample selection:

Where possible, animals of the same genotype, but of different cancer status were included in the study to investigate potential differences with regards to mRNA expression and disease state. In total 21 animals were entered into the study and their individual genotype, along with the cancer status of the animals is detailed in Table 5.1. The lower genital tract tissues were stored at The Marine Mammal Center (TMMC), Sausalito, California, at -80°C following necropsy examinations carried out by TMMC staff. The tissue samples were then shipped on dry ice to the Sea Mammal Research Unit (St Andrews, Fife) and on arrival they were stored at -80°C.

The experiments detailed in Chapter 3 identified *HPSE2* in the CSL; however it is not known whether this gene is transcribed in the lower genital tract of this species. In order to establish if this occurs and to identify any variation in the mRNA transcribed, cloning was undertaken. The process of cloning allows the amplification and subsequent sequencing of a single expressed gene fragment via its isolation in a single bacterial colony (Lodish et al., 2000). Examination of different colonies therefore enables the analysis of different expressed isoforms and/or polymorphisms within them.

To undertake cloning of *HPSE2* mRNA the following steps were carried out; (1) mRNA extraction and conversion to cDNA, (2) Confirmation of cDNA integrity, (3) Small amplicon PCR, (4) Full length amplicon PCR and gel extraction and purification, (5) Cloning and confirmation of positive clones, (6) Sequencing and isoform analysis.

Table 5.1. Pv11 genotype and cancer status of animals included in the *HPSE2* transcription study.

Accession No.	Pv11 genotype	Cancer/Control
9184(14)	1,1	Control
9325(76)	1,1	Control
9114(2)	1,1	Control
9303(36)	1,1	Control
9463(28)	1,1	Control
7972(74)	1,1	Cancer
7997(68)	1,1	Cancer
8431(69)	1,1	Cancer
9757(39)	1,1	Cancer
9827(41)	1,1	Cancer
9911(34)	1,1	Cancer
7819(75)	2,2	Control
9597(43)	2,2	Control
9724(25)	2,2	Cancer
9770(42)	2,2	Cancer
7867(73)	3,3	Cancer
9339(70)	3,3	Cancer
9572(71)	3,3	Cancer
8059(30)	3,3	Cancer
9821(72)	1,2	Control
9906(26)	1,2	Control

(1) mRNA extraction and conversion to complimentary DNA (cDNA)

RNA extraction and preparation

Total RNA was extracted using RNeasy Mini extraction Kit (Qiagen, Crawley, UK). The protocol was modified slightly from the manufacturer's instructions and is detailed below. The steps where the protocol deviates from the manufacturer's instructions are indicated in brackets at the relevant step. They include increased centrifugation times and additional incubation steps. The increased centrifugation times were employed as it was found that the ones in the protocol were insufficient to remove the fluid from the

spin column. The additional incubation steps where included in order to reduce contamination in the case of the incubation with buffer RW1 and to increase RNA elution in the RNase free water in the final steps. For all centrifugation steps the centrifuge was heated to 20-25°C. Extractions were not carried out in batches; instead each extraction was completed on its own to prevent any delays between the steps or the chance of contamination. New pipettes were purchased for the RNA extractions and filter tips were used at all times. In addition RNase free Eppendorf tubes were used. Between extractions the lab bench was cleaned down with UltraClean Lab Cleaner (Cambio, Cambridge, UK) and the homogeniser was cleaned thoroughly with RNase AWAY reagent (Invitrogen, Paisley, UK) to prevent nuclease and DNA contamination. The buffer RLT was prepared by the addition of 450 µl 14.3 M β-Mercaptoethanol (Sigma-Aldrich, Gillingham, UK) and buffer RPE were prepared by adding 44 ml of ethanol (100%).

Frozen tissue (10-30 mg) was excised and put into a 15 ml falcon tube as quickly as possible to avoid thawing and RNA degradation. Immediately, 600 µl of RLT buffer was added to the tube and the tissue homogenised. The homogenised tissue was transferred to a 1.5 ml Eppendorf and centrifuged at 17,000 x (*g*) for 3 min. The supernatant was added to 600 µl of 70% ethanol at 37°C and immediately mixed by pipetting. An RNesay spin column was placed into a 2 ml collection tube and up to 700 µl of the sample was added to the spin column. The sample was centrifuged for 1 min (increased from 15 s) at 14,500 x (*g*) and the flow through discarded; this was repeated with any remaining mixture followed by the addition of 700 µl of buffer RW1 to the spin column. The sample was incubated for 5 min (additional incubation step) at room temperature (15-25°C) then centrifuged for 1 min (increased from 15 s) at 14,500 x (*g*). The flow through was again discarded. The spin column was then washed by the addition of 500 µl of buffer RPE and centrifuged at 14,500 x (*g*) for 1 min (increased from 15 s), followed by a second wash step by the addition of 500 µl of buffer RPE and centrifuged for 2 min at 14,500 x (*g*). The RNeasy spin column was then placed in a new 2 ml collection tube and the old collection tube discarded with the flow through. The tube was centrifuged at 17,000 x (*g*) for 2 min (increased from 1 min). The RNeasy spin column was then placed in a new 1.5 ml collection tube and 30 µl of RNase-free water (Qiagen, Crawley, UK) was added directly to the spin column membrane. This

was incubated for 5 min (additional incubation step) at room temperature (15-25°C) followed by centrifuging it at 14,500 x (*g*) for 3 min (increased from 1 min). The elute was pipetted back into the spin column and the tube incubated at room temperature for (15-25°C) for 10 min (additional incubation step) before a final centrifugation step at 14,500 x (*g*) for 5 min (increased from 1 min). The quantity of RNA in the extraction samples was measured with a Nanodrop spectrophotometer (ND-1000, Thermo Fisher Scientific, Wilmington, USA), the samples were then stored at -80°C. Prior to storage 5 µl of extracted total RNA sample was removed and transferred on ice to a different lab for gel electrophoresis. The samples were resolved on 1.2% agarose gels for 15 min at 80 V.

Conversion to complementary DNA (cDNA)

To enable PCRs on the extractions the mRNA was converted to complementary DNA (cDNA) via reverse transcription using an Invitrogen Superscript III Reverse transcriptase kit (Invitrogen, Paisley, UK).

Each component in the kit was mixed and briefly centrifuged before use and 5 µg of total RNA along with 1 µl of 50 µM oligo(dt)20 and 1 ul of annealing buffer were added to a 0.2 ml PCR RNase free tube in that order and made up to 8 µl with RNase free water (Qiagen, Crawley, UK). The mixture was incubated in a preheated thermal cycler (PTC-200 DNA Engine Cycler, Bio-Rad Laboratories Inc., Hercules, USA) with the heated lid turned on, at 65°C for 5 min. Immediately after the 5 min the tubes were placed on ice for at least 1 min before brief centrifugation. The tubes were placed back on ice and 10 µl of 2X First-Strand reaction mix and 2 µl of SuperScript III/RNaseOUT Enzyme mix was added. The tubes were briefly vortexed then centrifuged to collect the sample. The mixture was then incubated in the thermocycler for 50 min at 50°C following which the reaction was terminated by heating it to 85°C for 5 min before chilling on ice and storing at -20°C.

(2) Confirmation of cDNA integrity

To confirm the integrity of the RNA extracted and resultant cDNA, PCR reactions were carried out using primers targeted to the mammalian beta actin gene (*ACTB*). *ACTB* is considered a housekeeping gene, its expression being important for correct cellular

function due to production of a cytoskeletal structural protein called beta actin (β actin) (Ng et al., 1985, Daud and Scott, 2008). The assumed continuous expression of housekeeping genes make them useful as internal controls to target when validating the quality of extracted RNA (Lee et al., 2002, Eisenberg and Levanon, 2013). However β actin levels vary amongst different tissues and its use has been questioned (Ruan and Lai, 2007, de Jonge et al., 2007), nonetheless it has been used successfully in female genital tissues in human studies (Baijal-Gupta et al., 2000, Arenas-Hernandez and Vega-Sanchez, 2013). Furthermore it has been used as an internal control previously in marine mammal studies therefore it was considered suitable for this study (Smolarek-Benson, 2005). Primers were obtained from Invitrogen (Paisley, UK) and the primer sequences are detailed in Table 5.2.

Table 5.2. Primer sequences for β-actin PCR

Primer	Sequence 5'-3'	Reference
BAF	GAGAAGCTGTGCTACGTCGC	(Smolarek-Benson, 2005)
BAR	CCAGACAGCACTGTGTTGGC	(Smolarek-Benson, 2005)

The PCR reaction used *Taq* DNA polymerase (New England Biolabs, Hitchin, UK) with thermopol buffer (New England Biolabs, Hitchin, UK) in 25 µl reaction mixes consisting of 2.5 µl reaction buffer, 0.5 µl 10 mM dNTPs (Qiagen, Crawley, UK), 0.5 µl BAF forward primer (10 µM), 0.5 µl BAR reverse primer (10 µM), 0.125 µl *Taq* (0.625 units) and 18.375 µl RNAse free water (Qiagen, Crawley, UK). 2.5 µl of unquantified cDNA was added as template. Amplification was carried out in a PTC-200 DNA Engine Cycler (Bio-Rad Laboratories Inc., Hercules, USA) using the following cycle; 95°C for 30 s followed by 35 cycles of 95°C for 30 s, 53°C for 30 s, 68°C for 60 s. The samples were then held for 5 min at 68°C before being stored at 4°C. Confirmation of successful conversion to cDNA was made by resolving the products on a 1.5% agarose gel with an expected product size of 275 bp. If unsuccessful the reactions were repeated with increased primer and *Taq* concentration (1 µl 10 µM BAF and BAR along with 0.2 µl *Taq* (1 unit) respectively). A negative control was included by substituting the cDNA template with the same volume of RNAase free water (Qiagen, Crawley, UK) and a positive control included by amplifying previously verified cDNA.

(3) Small amplicon PCR

To confirm the presence of *HPSE2* mRNA in the samples a hemi-nested PCR reaction was carried out to generate a small fragment. The primer sequences used were previously designed and validated for the purpose of this study (Hammond, unpublished), and were obtained from Invitrogen (Paisley, UK). Primer sequences are detailed in Table 5.3 and their position relative to the structure of the *HPSE2* gene is illustrated in Figure 5.1. 25 µl reactions were used consisting of 5 µl Go Taq flexi colourless buffer (Promega, Southampton, UK), 2.5 µl $MgCl_2$ (25 mM), 0.5 µl 10 mM dNTPs (Qiagen, Crawley, UK), 1.5 µl forward primer (10 µM), 1.5 µl reverse primer (10 µM), 0.125 µl Go *taq* polymerase (0.625 units) (Promega, Southampton, UK), 12.875 µl RNase free water (Qiagen, Crawley, UK). 1 µl of un-quantified cDNA was used as the template. The primary reaction cycle carried out in a PTC-200 DNA Engine Cycler (Bio-Rad Laboratories Inc., Hercules, USA) was as follows; 94°C for 2 min followed by 35 cycles of 94°C for 20 s, 56°C for 20 s and 72°C for 30 s, the mixtures were then held at 72°C for 7 min before being stored at 4°C. A 1:5 dilution of the PCR product was made by adding 5 µl of product to 20 µl of RNase free water (Qiagen, Crawley, UK) and 1 µl of this was then used as the template for the nested reaction. The reaction mix was the same as the primary mix but the reaction cycle was modified by reducing the cycle number to 30. A negative control was included in both reactions by substituting the template with RNase free water (Qiagen, Crawley, UK). The presence of *HPSE2* isoform was identified by resolving 5 µl of product of the secondary PCR combined with 2.5 µl Orange G loading dye (Sigma-Aldrich, Ltd, Gillingham, UK) on a 1.5% gel containing 2.5 µl of 10 mg/ml ethidium bromide (Sigma-Aldrich Ltd, Gillingham, UK). The expected product size was 159 bp.

Table 5.3: Primer sequences used the PCR to amplify a for small fragment of *HPSE2*.

Primer	Sequence 5'-3'	Reference
Primary PCR		
105_S	F: ATG CCC TCC AGC AAC TCC	(Hammond, unpublished)
358_{AS}	R: AAT CGA GCC AGC CAT CAT G	
Nested PCR		
199_S	F: GAG ACA GGA GAC CCT TGC C	(Hammond, unpublished)
358_{AS}	R: AAT CGA GCC AGC CAT CAT G	

(4) Full length amplicon PCR

A hemi-nested PCR was carried out to amplify the full length *HPSE2* isoform in six of the 21 samples of different genotype and disease state; these are detailed in Table 5.4. The primers used were previously designed and validated for the purpose of this study (Hammond, unpublished), the primers were obtained from Invitrogen (Paisley, UK) and the sequences are detailed in Table 5.5 and their position relative to the structure of the HPSE2 gene is illustrated in Figure 5.1. The primary PCR reaction consisted of 25 µl reaction mixtures of 2.5 µl 10xNH4 buffer (Bioline Reagents Ltd, London, UK), 1.25 µl 50 mM $MgCl_2$ (Bioline Reagents Ltd, London, UK), 0.5 µl 10 mM dNTP (Qiagen, Crawley, UK), 1 µl (10 µM) forward primer (HPSE2_5'UTR-S), 1 µl (10 µM) reverse primer (HPSE2_3'UTR-AS2), 0.25 µl Bio-X-ACT short DNA polymerase (1 unit) (Bioline Reagents Ltd, London, UK), 17.5 µl RNase free water (Qiagen Crawley, UK) and 1 µl of un-quantified cDNA as template. Amplification was carried out in a PTC-200 DNA Engine Cycler (Bio-Rad Laboratories Inc., USA) and was as follows; 94°C for 2 min, followed by 35 cycles of 94°C for 25 s, 57°C for 20 s, 72°C for 2 min 30 s, before being held at 72°C for 7 min and then stored at 4°C.

For the nested reaction a 1:5 dilution of the PCR product of the primary reaction was made and 2 µl of this was used as template DNA. 50 µl reaction mixtures were used and five reactions for each sample were prepared to give greater yield of product for subsequent cloning. The reactions consisted of; 5 µl 10xNH4 buffer (Bioline Reagents Ltd, London, UK), 2.5 µl 50 mM $MgCl_2$ (Bioline Reagents Ltd, London, UK), 1 µl 10 mM dNTP (Qiagen, Crawley, UK), 2 µl 10 mM forward primer (HPSE2_5'UTR-S2), 2 µl 10 mM reverse primer (HPSE2_3'UTR-AS2), 0.5 µl (2 units) Bio-X-ACT short DNA polymerase (Bioline Reagents Ltd, London, UK) and 35 µl RNase free water (Qiagen, Crawley, UK). The reaction cycle was modified by reducing the cycle number to 30. A negative control was included in both reactions by substituting the template with RNase free water (Qiagen, Crawley, UK). The products of the five reactions per sample were each combined with 5 µl Orange G loading dye (Sigma-Aldrich, Gillingham, UK) and resolved side by side at 80 V on a 30 cm 1.2% agarose gel. The gel was post stained by soaking it in 1 litre of distilled water containing 15 µl of 10

mg/ml ethidium bromide (Sigma-Aldrich Ltd, Gillingham, UK) for 20 min, followed by soaking the gel in 1 litre of distilled water prior to visualising it in a UV light box (UVITEC, Cambridge, UK). The product size of the secondary PCR was 1870 bp.

Table 5.4. Pv11 genotype and disease state of animals used to investigate *HPSE2* isoforms

Accession No.	Genotype	Cancer/Control
9463(28)	1,1	Control
7972(74)	1,1	Cancer
9770(42)	2,2	Cancer
9339(70)	3,3	Cancer
9572(71)	3,3	Cancer
9821(72)	1,2	Control

Table 5.5. Primer sequences used in the PCR to amplify the full length *HPSE2* isoform

Primer	Sequence 5'-3'	Reference
Primary PCR		
HPSE2_5'UTR-S	F:ATC AGA GGG ATT TAA TGA GGG TG	(Hammond,
HPSE2_3'UTR –AS2	R:CAT GGT GAC TGG AGG GAT GAC	unpublished)
Nested PCR		
HPSE2_5'UTR-S2	F:ATG AGG GTG CTC TGT GCC TTC	(Hammond,
HPSE2_3'UTR –AS2	R:CAT GGT GAC TGG AGG GAT GAC	unpublished)

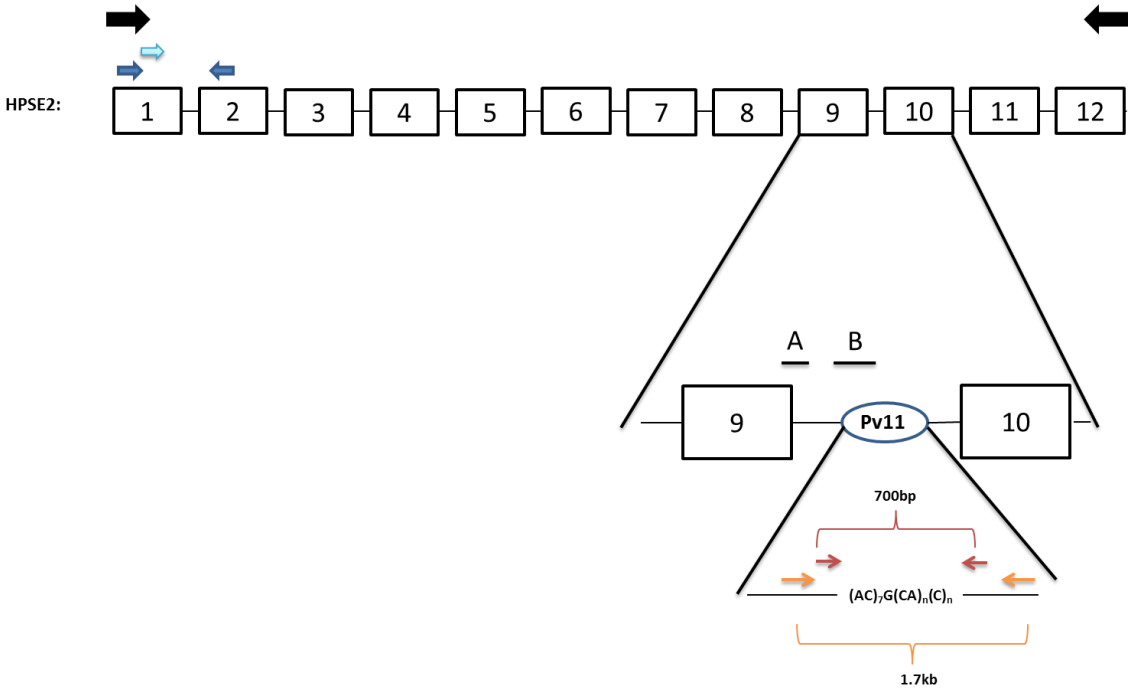


Fig 5.1: Structure of the *HPSE2* gene is conserved amongst mammals and consists of 12 exons separated by large intronic regions. The first pop out window illustrates the position of the two probes (A and B) employed in the southern blot. The second pop out window illustrates the position of the two sets of primers used to amplify the Pv11 region prior to sequencing the microsatellite. The three blue arrows situated above exons 1 and 2 illustrate the position of the primers used in the hemi-nested PCR carried out to amplify a small fragment of the *HPSE2* gene. The dark blue arrows indicate the primer positions for the primary PCR and the light blue arrow indicates the position of the forward primer in the secondary PCR. The two large black arrows situated above exons 1 and 12 indicate the position of the primers used for amplify the full length amplicon.

(5) Cloning and confirmation of positive clones

Identification of isoforms following PCR was achieved via cloning and subsequent sequencing, the method undertaken is detailed in full in Appendix D. Following sequencing initial identification of the presence of *HPSE2* isoforms was carried out using the online NCBI Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). The sequences were then visualised using the software programme Geneious Pro v5.6.6 (Biomatters, available from http://www.geneious.com/). The primer regions were removed along with trimming of end sequences of low quality. This was followed by a multiple alignment, of the sequences to allow the identification of different isoforms. Any isoforms identified were then mapped to the canine full length isoform *HPSE2* cDNA sequence scaffold (consisting of all 12 exons) in order to visualise their structure.

5.2.2 [2]*HPSE2* Translation

Sample selection

Formalin fixed paraffin embedded blocks containing tissues including lower genital tract tissues from 15 animals (previously admitted to TMMC) were supplied by Dr Kathleen Colegrove (University of Illinois, Chicago, USA). The samples included six UGC negative controls and nine UGC positive animals. The samples affected by neoplasia were graded according to their disease stage by Dr Kathleen Colegrove as previously described (Colegrove et al., 2009).

In order to investigate *HPSE2* expression at the protein level in lower genital tract tissue immunohistochemistry (IHC) was carried out. IHC allows the visual detection of an antigen (protein) of interest, in this case HPA2, by utilizing labelled antibodies specifically raised against it (Haines and Chelack, 1991). The IHC protocol undertaken in this study employed the avidin-biotin complex (ABC) method which increases the chance of detection due to increased amplification of the chromogen signal (Haines and Chelack, 1991).

[2] The IHC protocol was carried out in collaboration with Dr Mark Dagleish's Lab at The Moredun Research Institute, Edinburgh, UK

The steps employed in the study were as follows; (1) Sample preparation, (2) Antigen retrieval, (3) Blocking, (4) Application of antibodies, (5) Detection and (6) Slide analysis.

(1) Sample Preparation

Two consecutive serial sections (4 µm) were cut per tissue, to allow the inclusion of a negative control for each sample, and mounted on Superfrost™ slides (Menzel-Gläser, Braunschweig, Germany) by Ms Jeanie Finalyson (The Moredun Institute, Edinburgh, UK). Sections were dewaxed in xylene and rehydrated through graded alcohols in a Varistain™ 24-4 Automatic Slide Stainer (Thermo Scientific, Waltham, USA).

(2) Antigen Retrieval

The success of an IHC reaction relies on the antibody being able to recognise its antigen. Prior to embedding in paraffin wax, tissue samples are preserved in formaldehyde. This results in protein cross linking that can mask antigenic sites (Ramos-Vara, 2005, Sutherland et al., 2008). Antigen retrieval is required to reverse the cross-linking and can be achieved both by heating the sample and the use of enzymes. In this case enzymatic antigen retrieval was carried out by treating the samples with trypsin. 0.1 g of Chymotrypsin (Sigma-Aldrich Ltd, Gillingham, UK) was added to 200 ml trypsin working solution (20 ml 0.1 M $CaCl_2$, 20 ml 0.5% trypsin stock solution, 160 ml purified water at pH 7.8). The solution was heated to 37°C and the slides incubated for 15 min followed by washing in running tap water for 5 min.

(3) Blocking

Blocking of three elements in the tissues was carried out to prevent non-specific background labelling. These were endogenous tissue peroxidase (which can react with the chromogen used for detection), non-specific antibody binding sites and endogenous biotin which can also result in non-specific binding (Ramos-Vara, 2005, Haines and Chelack, 1991). Blocking endogenous tissue peroxidase activity was carried out by immersion in H_2O_2 in methanol (3% v/v) for 20 min. Sections were then washed in running tap water for 5 min. The slides were then put into coverplates using phosphate

buffered saline (PBS) and loaded into Sequenza chambers for the rest of the process (Figure 5.2), prior to being immersed in 25% normal rabbit serum (NRS) diluted in 0.33 M pH 7.4 PBS for 30 min at room temperature to block non-specific antibody sites. This was followed by blocking of endogenous tissue biotin binding with a commercial kit (Avidin/Biotin blocking kit, (Vector Laboratories, Peterborough, UK) as per manufacturer's instructions.

Fig 5.2 Immunohistochemistry Sequenza chamber containing slides of lower genital tract tissue sections.

(4) Application of antibodies

The primary antibody used in this study was a polyclonal, goat IgG raised against a peptide of human heparanase 2 (HPA2, C-17, Santa Cruz Biotechnology, Inc. Santa Cruz, USA). It is reported to target a peptide near to the C terminus and is reported in the data sheet as suitable for detecting HPA2 in species other than humans including canine. The primary antibody was diluted 1:100 in 25% NRS/PBS and 100 µl applied before being incubated in the fridge at 4°C overnight. A negative control preparation for each of the tissue sections comprised of substituting the primary antibody with normal goat serum at a dilution of 1:100. Slides were rinsed in PBS three times prior to addition of the secondary antibody. This was a rabbit anti-goat IgG:biotin conjugate (Dako, Ely, UK) diluted 1:200 in 25% NRS/PBS, 100 µl of which was applied to the slides for 60 min at room temperature, followed by a further three washes in PBS.

(5) Detection

The application of the secondary biotinylated antibody allows the binding of an avidin protein, which itself can bind four molecules of biotin resulting in an avidin biotin

complex (ABC). The biotin in the ABC avidin biotin kit (Vector Laboratories, Peterborough, UK) used in this study is labelled with horseradish peroxidase (HR). HR can act on a chromogen substrate resulting in a visible label, which is subsequently amplified due to the formation of ABC complexes arising from a single antigen-antibody interaction (Ramos-Vara, 2005, Haines and Chelack, 1991). The ABC kit was prepared by adding 100 µl of solution A (containing avidin) and 100 µl of solution B (containing biotinylated horseradish peroxidase) to 10 ml PBS and immediately mixing. The ABC complex was incubated at room temperature for 30 min before 100 µl of it was applied to the slides. The slides were incubated at room temperature for 30 min before being rinsed three times with PBS. The liquid was emptied from the Sequenza chambers prior to the application of the chromogen NovaRED (Vector Laboratories, Peterborough, UK). The NovaRED was prepared according to manufacturer's instructions and 100 µl was applied to the slides and they were incubated for 10 min at room temperature. The slides were then washed with tap water and removed from the Sequenza chamber before being counter stained with haematoxylin in the Varistain™ 24-4 Automatic Slide Stainer (Thermo Scientific, Waltham, USA). Coverslips were then applied and the slides left to dry.

(6) [3]Slide analysis

Sections were observed using an Olympus BX50 microscope at magnifications of x200 or x400 and photographed using an Olympus U-CMAD digital camera and AnalySIS Five software (Soft Imaging System GmbH, Münster, Germany). The presence of labelling was compared between the UGC positive and UGC negative samples as well as examination of the negative control slides (where normal goat serum was substituted for the primary antibody). The samples were scored "yes" if clear labelling was present and "no" if labelling was absent. In ambiguous cases findings were noted. Additional tissues (other than those from the lower genital tract) incidentally on the slides were also reviewed for the presence of labelling; these included three sections of urinary bladder and two sections of uterus. In the absence of clear information on the normal protein expression profile of HPA2 in tissues, the uterus and urinary bladder sections

[3] Interpretation of histology slides was undertaken with the assistance of Dr Mark Dagleish and Ms Johanna Baily, The Moredun Research Institute, Edinburgh, UK and Dr Kathleen Colegrove, Veterinary Diagnostic Laboratory, University of Illinois, USA.

were considered as potential positive control tissues due to previous reports of high mRNA expression in these tissues.

5.2.3 Problems encountered

During the investigation of *HPSE2* transcription three main issues were encountered; (1) the samples were held up in transit from the USA and on arrival were found to be defrosted and there was a concern that RNA degradation had occurred, (2) the gel rigs and pipettes used for gel electrophoresis to analyse the extracted and purified total RNA were not specific for RNA work. Therefore there was a high chance of nuclease contamination and therefore degradation of the samples, additionally degradation of the samples during transfer on ice to the other lab was possible (3) Ligating the insert into a vector prior to transformation into TOP10 cells (Invitrogen, Paisley, UK) initially was unsuccessful. The ligation vector employed initially was the TOPO TA vector for sequencing (TOPO®TA Cloning ® Kit for Sequencing, Invitrogen, Paisley, UK). Experiments using the control reaction and subsequent insert of size 750 bp were successful, however ligation of the insert created by this study (of approximately 1870 bp) consistently failed. The literature available suggested that the insert size was the issue and that TOPO vector works well for inserts of less than 1 kb, whereas success was reduced with inserts of greater than this. The pGEM T-easy vector however was observed to work well with inserts up to 3 kb (Litterer, 2009), therefore this vector was used instead.

Identifying the presence of HPA2 was complicated by the lack of a definite positive control tissue as a result of the small number of studies that have been undertaken on HPA2 presence in tissue.

5.3 Results

5.3.1 *HPSE2* gene transcription

Extraction of total RNA was successful from all 21 samples with quantities between 53.4 ng/µl and 400.8 ng/µl recorded. Gel electrophoresis revealed a varied banding pattern with the majority revealing only a single band (Figure 5.3). This was possibly due to degradation of the samples mentioned above.

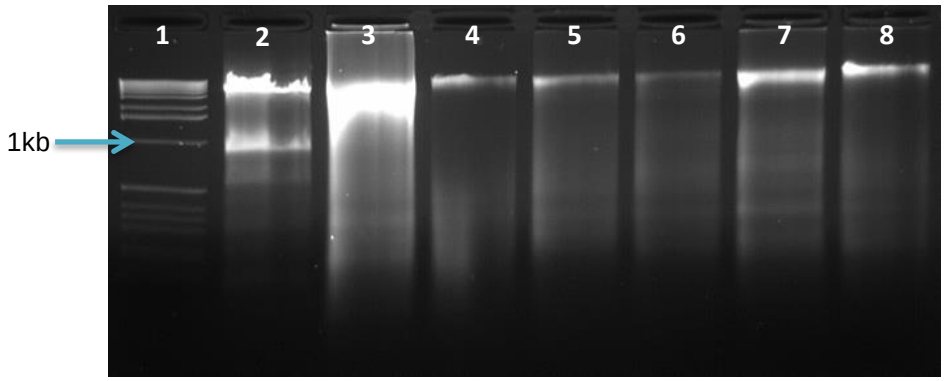


Fig. 5.3: Example of gel electrophoresis following total RNA extraction. Lane 1] 1kb ladder, 2] 9184(14), 3] 9770(42), 4] 8431(69), 5] 8059(30), 6] 7819 (75), 7] 7867(73), 8] 9114(2)

In the β actin PCR to confirm cDNA integrity, 19 out of the 21 reactions were clearly successful (Figure 5.4). PCRs were repeated on the two unsuccessful samples utilizing the higher primer and *taq* concentrations. On gel electrophoresis one sample (8431(69)) still did not reveal successful amplification of the β actin fragment.

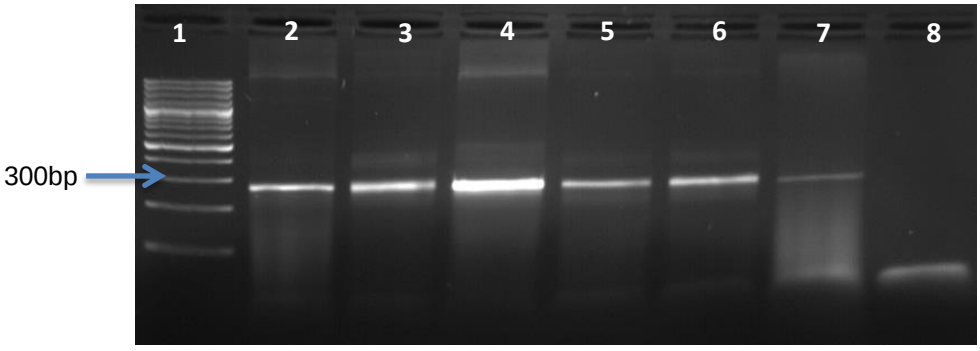


Fig 5.4. Example of gel electrophoresis of products of β actin PCR to assess cDNA integrity. Bands of appropriate size (approx. 275bp) are seen. Lane 1] 100bp ladder, 2] 9911(34), 3] 7867(73), 4] 9339(70), 5] 9572 (71), 6] 8059 (30), 7] Positive control, 8] Negative control (showing evidence of primer dimers >100bp)

Small amplicon PCR

Initially a small fragment (159 bp) of *HPSE2* cDNA was amplified during a hemi-nested PCR to confirm the presence of *HPSE2* mRNA in the samples (Figure 5.5). Clear amplification was identified in 18 of the 21 samples however three revealed only weak amplification, including sample 8431(69) which as noted above failed to amplify a β actin fragment.

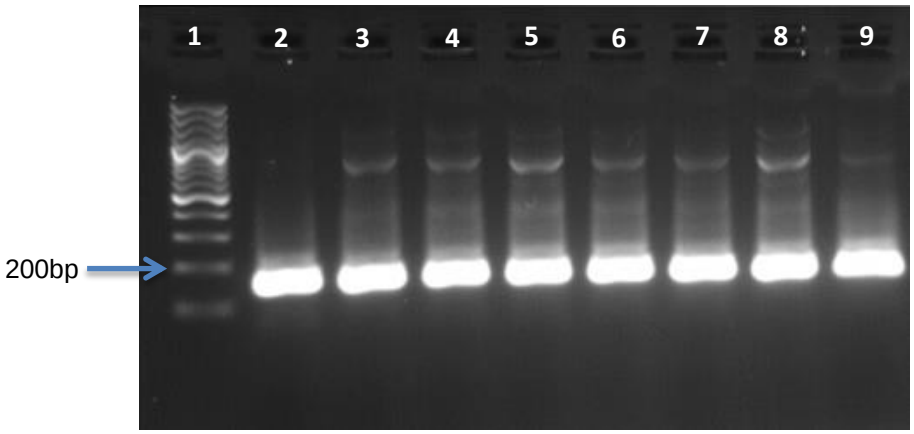


Fig 5.5 Example of gel electrophoresis of hemi-nested PCR result revealing amplification of a small fragment of the HPSE2 gene (expected band size of 159bp). Lane 1] 100bp ladder, 2] 9184(14), 3] 9325(76), 4] 9114(2), 5] 9303(36), 6] 9463(28), 7] 9906(26), 8] 7972(74), 9] 7997(68).

Large amplicon PCR

Six samples were used to amplify the full length amplicon. The six samples were chosen due to their differing genotype and disease state. Each PCR being repeated five times to allow sufficient quantity for subsequent cloning of each isoform. In all six samples the expected multiple banding pattern was visualised by gel electrophoresis. An example of the banding pattern for the four different Pv11 genotypes is shown in Figure 5.6. There were variations visualised in the banding pattern from the five repeated PCR reactions carried out on the same DNA template, however a comparison of the overall banding pattern between the different animal samples did not reveal any correlation with disease state or genotype. Therefore only the three dominant bands closest to the expected product size of approximately 1870 bp were extracted for cloning and sequencing (Figure 5.6).

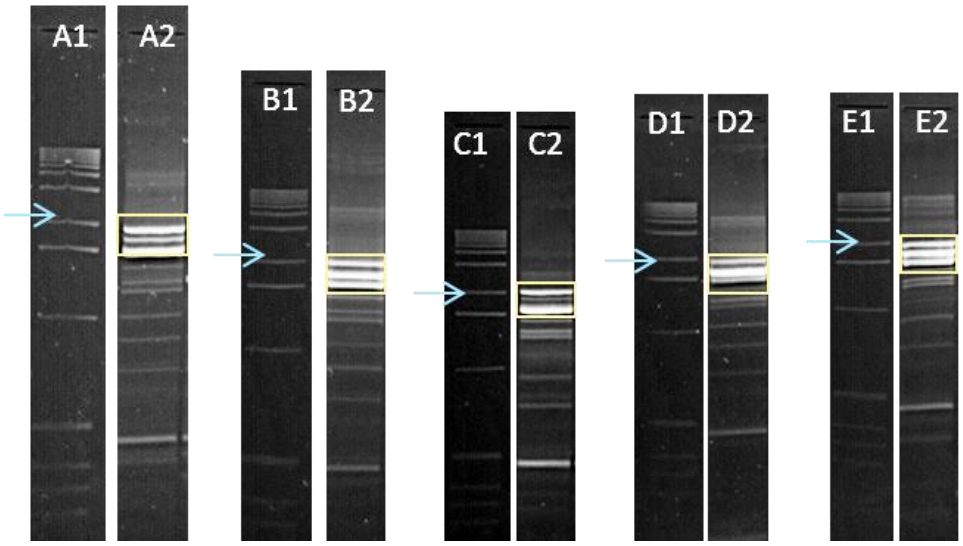


Fig 5.6. Gel electrophoresis of five of the isoform PCRs illustrating multiple banding patterns from animals of different disease states and genotype. The expected product size of the full length isoform is ~1870bp, the blue arrows indicate the 2kb marker associated with each gel and the orange box illustrates the bands that were extracted for cloning. A1, B1, C1, D1 and E1 illustrated the corresponding 1kb marker pattern. A2: Banding pattern from PCR amplification of DNA from animal 9463(28), a control allele one homozygote. B2: Banding pattern from PCR amplification of DNA from animal 7972(74), a UGC allele one homozygote. C2: Banding pattern from PCR amplification of DNA from animal 9770(42), a UGC allele two homozygote. D2: Banding pattern from PCR amplification of DNA from animal 9572(71) a UGC allele three homozygote and E2: Banding pattern from PCR amplification of DNA from animal 9821(72) a control allele one/two heterozygote.

Cloning and sequencing

Cloning success varied amongst the samples with six to 12 out of the 16 colonies taken resulting in positive plasmid preparations on digestion, identified as bands of the appropriate insert size on gel electrophoresis following the digestion reaction (Figure 5.7). Sequencing of positive clones revealed the presence of five isoforms, four produced via intron deletions and a fifth truncated miss-spliced isoform (Table 5.6 and Figure 5.8). The vast majority of the clones sequenced were the full length isoform (isoform one) which corresponded to canine isoform two, and isoform three which corresponded to canine isoform four. On examination of the sequences no polymorphism was identified between any of the products or animals in the study.

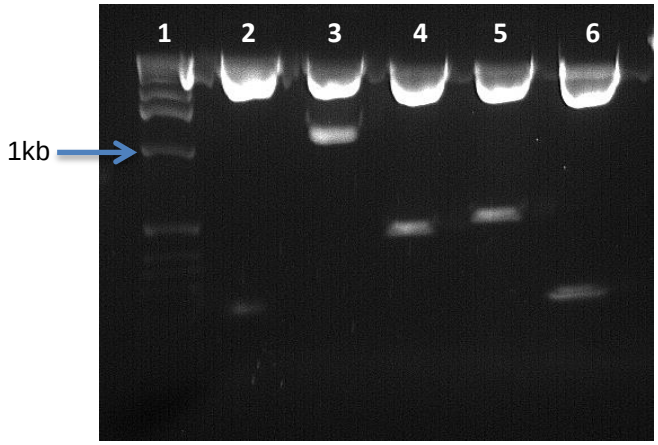


Fig 5.7 Gel electrophoresis of an example of one positive plasmid prep following restriction digest (Lane 3), the other lanes (2, 4-6) show inserts of clearly the wrong size. Lane1] 1kb ladder.

Table 5.6 Isoforms identified with corresponding disease state along with Pv11 genotype of the animals the isoforms were obtained from, size of isoform and spliced structure of isoform.

Isoform	Size(bp)	Structure	Disease state	Pv11 genotype	Animal ID
One	1686	Full length	Control	1,1; 1,2	9463(28); 9821(72)
One	1686	Full length	UGC positive	1,1 ; 2,2; 3,3	7972(74); 9770(42); 9572(71)
Two	1512	Exon 4 out	Control	1,2	9821(72)
Three	1525	Exon 3 out	UGC positive	2,2; 3,3	9770(42); 9339(70)
Four	1571	Exon 9 out	Control	1,1	9463(28)
Five	728	Miss-spliced	UGC positive	1,1	7972(74)

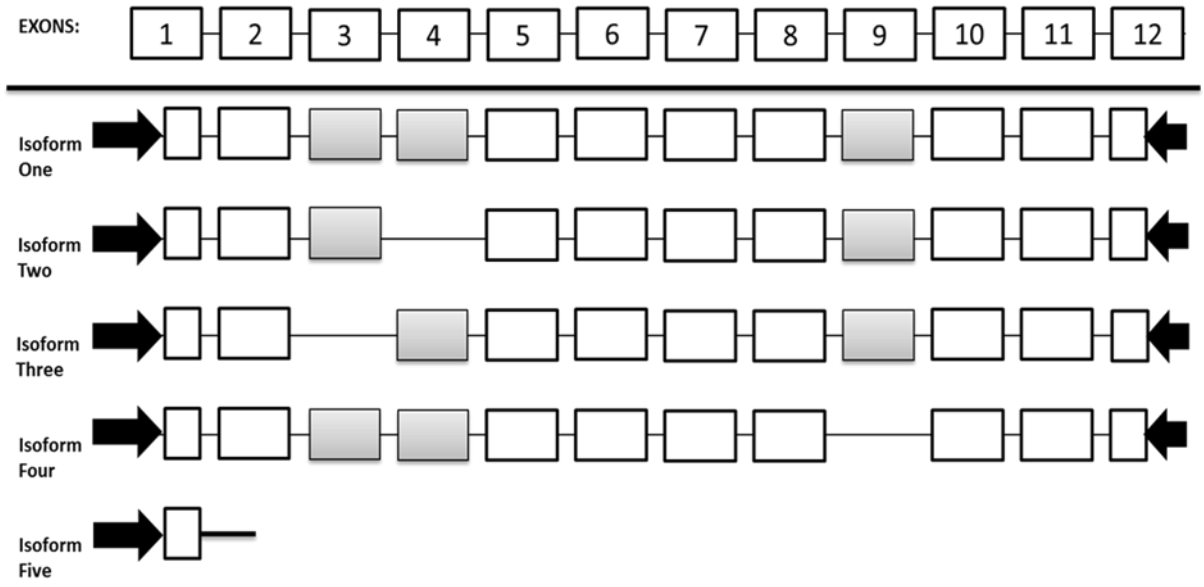


Fig. 5.8. Structure of the five identified CSL isoforms highlighting the variable spliced exons. Exons are depicted as boxes and intron regions and spliced exons as the thin lines connecting them. In cases of miss-splicing the intron line is represented as a thick line indicating some intron sequence present. The 12 exons of *HPSE2* are at the top of the diagram. Primer sites are indicated by the arrows. Isoform one was isolated from both control and UGC animals is the full length isoform containing all of the exons; Isoform two was isolated from a control animal and has exon 4 spliced out; Isoform three was isolated from UGC animals has exon 3 spiced out; Isoform four was isolated from a control animal and has exon 9 spliced out; Isoform five is comprised of exon 1 and intron 1 sequences due to miss-splicing.

The diagram illustrated in Figure 5.9 gives an overview of the investigations carried out on the *HPSE2* gene in the present study. It includes positions of the probes used in the southern blot (Chapter 3), position of primers used in amplifying the Pv11 microsatellite for sequencing (Chapter 4) along with the position of the primers used to amplify the isoforms during the experiments detailed in this Chapter. The diagram also indicates the exons which were identified as alternatively spliced on examination of the isoform sequences.

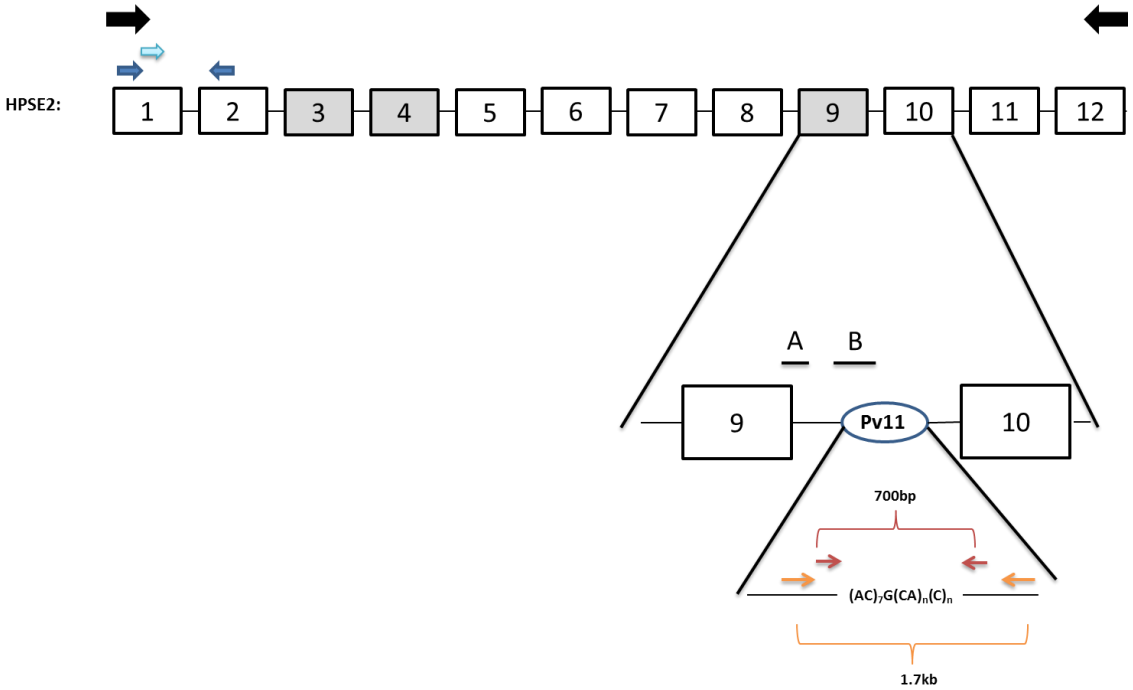


Fig 5.9: Structure of the *HPSE2* gene is conserved amongst mammals and consists of 12 exons separated by large intronic regions. The first pop out window illustrates the position of the two probes (A and B) employed in the southern blot. The second pop out window illustrates the position of the two sets of primers used to amplify the Pv11 region prior to sequencing the microsatellite. The three blue arrows situated above exons 1 and 2 illustrate the position of the primers used in the hemi-nested PCR carried out to amplify a small fragment of the *HPSE2* gene. The two large black arrows situated above exons 1 and 12 indicate the position of the primers used for amplify the full length amplicon. Exons 3, 4 and 9 shaded in grey were found to be alternatively spliced on examination of isoform sequences.

5.3.2 *HPSE2* translation

Identification of protein in lower genital tract tissue

IHC was undertaken to identify the presence of HPA2 in samples of lower genital tract tissue from 15 animals of differing Pv11 genotype. The animals were chosen due to the availability of suitably preserved tissue and consisted of nine UGC positive and six UGC negative animals. Four of the samples – 9463(28), 7972(74), 9770(42) and 9339(70), had additionally been included in the investigation into transcription of the *HPSE2* gene. Therefore it was known that *HPSE2* isoform mRNA was present in these tissues indicating that there was potential for the HPA2 protein to be present.

Of the 15 lower genital tract sections examined five (all UGC positive) were positive for HPA2. Interestingly, all the IHC positive tissues were from Pv11 allele one homozygote animals (Table 5.7 and Figure 5.10 and 5.11). In three of the positive samples labelling was seen within the neoplastic cells with the greatest amount in cells in the basal layer of the epithelium (Figure 5.10). In the remaining two IHC positive animals, labelling was present within the cytoplasm of neurons associated with the cervix and within mononuclear inflammatory cells within the cervix submucosa (Figure 5.11). There was no evidence of labelling in any of the other lower genital tract samples examined, including the UGC negative animals with a Pv11 one homozygote genotype, or the negative control samples (where the goat-anti human HPA2 polyclonal antibody was substituted with normal goat serum). Therefore, HPA2 presence is associated with animals with UGC of one homozygous Pv11 genotype, and confirms the link between *HPSE2* and UGC.

Table 5.7 Results of HPA2 immunolabelling of lower genital tract tissues of various Pv11 genotype and disease states. LGIL: Low grade intraepithelial lesion; HGIL: High grade intraepithelial lesion; IC: Invasive carcinoma. Numbers in bold indicate the samples where labelling was within neurons associated with the cervix tissue (8431(69)) and in inflammatory cells in the cervix tissue (9757(39)).

Pv11 genotype	UGC positive/control	animal ID	genital tissue labelling	genital tissue (lesion grade)
1,1	Control	9184(14)	Negative	Cervix
		9325(76)	Negative	Cervix +vagina
		9114(2)	Negative	Cervix+vagina
		9463(28)	Negative	Cervix
	UGC positive	7972(74)	Positive	Vagina (HGIL)
		7997(68)	Positive	Cervix (IC)
		8431(69)	Positive	Cervix (HGIL)
		9757(39)	Positive	Cervix (IC)
		9911(34)	Positive	Cervix (IC)
2,2	Control	7819(75)	Negative	Cervix+vagina
	UGC positive	9724(25)	Negative	Cervix (HGIL)
		9770(42)	Negative	Cervix+vagina (LGIL)
3,3	UGC positive	7867(73)	Negative	Cervix (HGIL)
	UGC positive	9339(70)	Negative	Cervix+vagina (HGIL)
2,4	Control	9274(8)	Negative	Cervix

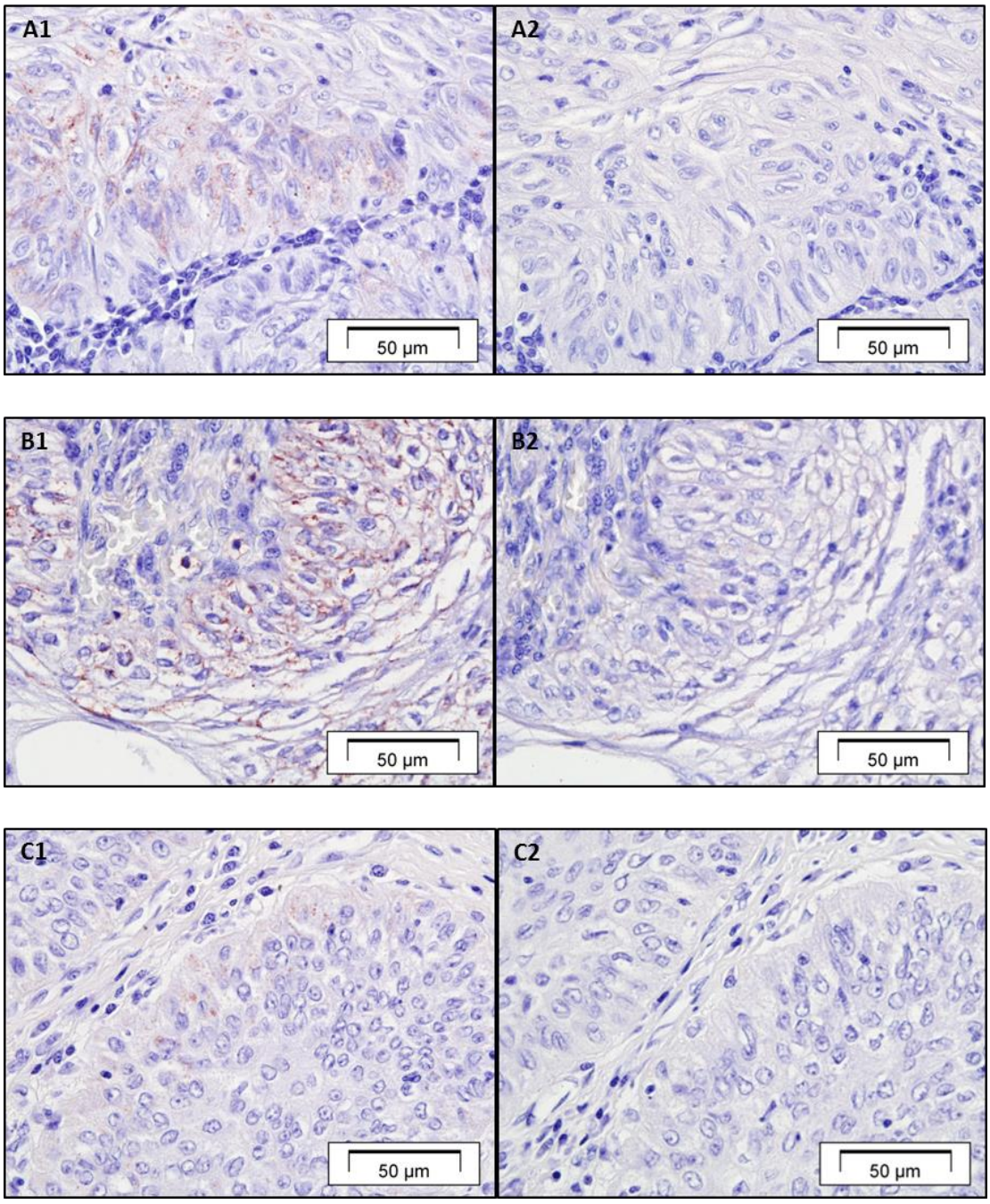


Fig 5.10. Positive immunolabelling of HPA2 in neoplastic lower genital tract tissue from three female CSLs of homozygous Pv11 genotype 1,1. Sequential negative control sections (A2, B2 and C2) are pictured on the right. A: Animal 7972(74) (vagina) B: Animal 7997(68) (cervix) C: Animal 9911(34) (cervix)

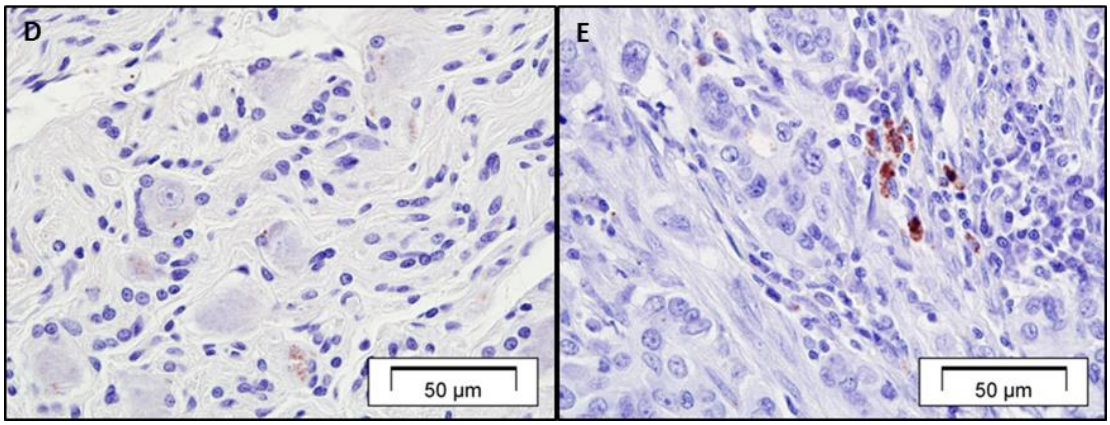


Fig 5.11. Positive immunolabelling of HPA2 in neoplastic lower genital tract tissue from two female CSLs of homozygous Pv11 genotype 1,1. Section D and E illustrate the unusual labelling pattern identified in animals 8431(69) and 9757(39) where cytoplasmic labelling was identified within neurons associated with the cervix and in mononuclear inflammatory cells in the cervix submucosa, respectively.

Identification of protein in other tissue

The tissue blocks obtained for the study incidentally contained tissues other than those from the lower genital tract. Due to the small number of studies that have identified expression of *HPSE2* at the protein level (Table 3.2), these tissues were examined for labelling in addition to the main study. Of particular interest being tissues from the genital tract, other than cervix and vagina. The tissues examined included uterus, ovary, heart, diaphragm, stomach, urinary bladder and lymph node. Of these tissues protein has only been reported previously in the ovary in cases of benign and malignant ovarian cancer (de Moura et al., 2009) and in the stomach in cases of gastric neoplasia (Zhang et al., 2013). Uterus and urinary bladder have been noted to show high levels of *HPSE2* mRNA (McKenzie et al., 2000, Pang et al., 2010). On examination of the samples labelling was only identified in the stomach (animal 9911(34), an allele one homozygote suffering from UGC). The findings are summarised in Table 5.8 and the positive stomach sample and an example of a negative uterus sample are illustrated in Figure 5.12.

Table 5.8. Results of tissues other than lower genital tract tissues examined for HPA2 immunolabelling. The only tissue that exhibited immunolabelling was in the stomach of cancer animal 9911(34)

Accession No.	Pv11 genotype	Other tissues	Cancer/Control	Labelling
9184(14)	1,1	Urinary bladder	Control	Negative
		Diaphragm		Negative
9463(28)	1,1	Ovary	Control	Negative
		Lymph node		Negative
		Heart		Negative
7972(74)	1,1	Urinary bladder	Cancer	Negative
		Heart		Negative
9911(34)	1,1	Stomach	Cancer	Positive
7819(75)	2,2	Lymph node	Control	Negative
9724(25)	2,2	Tonsil	Cancer	Negative
7867(73)	3,3	Ovary	Cancer	Negative
		Uterus		
9339(70)	3,3	Urinary bladder	Cancer	Negative
9274(8)	2,4	Ovary	Control	Negative
		Uterus		

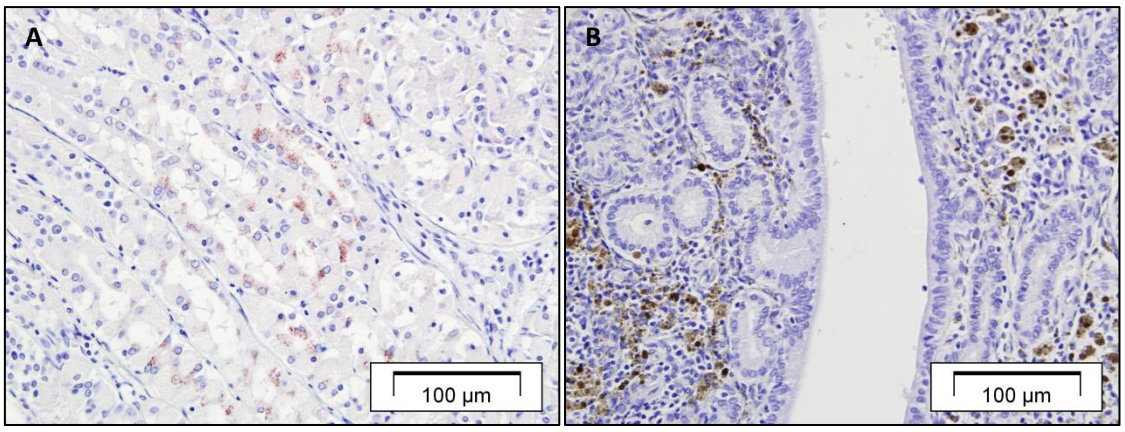


Fig 5.12. Immunohistochemistry sections of other tissues examined. A] Positive labelling in the stomach. B] Uterus with obvious haemosiderin deposits seen as the brown areas, but no labelling.

5.3 Discussion

The work carried out in previous chapters identified Pv11 as a microsatellite marker with a significant association with UGC in the CSL. Comparative genomics and further molecular work placed it within an intron of the large genetic locus *HPSE2*. The association discovered regarding Pv11 potentially indicates a QTL and the large size of *HPSE2* along with the reported sequence homology to the *HPSE* gene in humans made it a natural choice to investigate further. However the candidate gene responsible for the association identified may be at a location distant from Pv11 in the genome. In spite of this *HPSE2* offered a good starting point in investigating a genetic basis to the condition.

The findings of this study demonstrate that the *HPSE2* gene is expressed at least to the mRNA level in lower genital tract tissues of the female CSL of different Pv11 genotypes. In addition the presence of alternative splicing was confirmed by the identification of five splice variants. It is likely however that the true number of isoforms is greater than the five examined here as the resultant gels following PCR revealed multiple banding patterns. Further cloning and sequencing of each band is required in order to clarify this. No variation was detected between isoform sequences from animals with UGC compared to control animals; however it was not determined how much mRNA was present for each isoform detected. The use of quantitative techniques such as qPCR in future studies would assist in assessing if any differences in the quantity of mRNA for a particular isoform was occurring between cases and controls or amongst different genotypes. This possibility is supported by the labelling pattern identified where HPA2 was only expressed in animals suffering from UGC that were of a single homozygous genotype. In two of the samples the labelling pattern was unusual, with labelling identified in neurons associated with cervix tissue and within mononuclear inflammatory cells in cervix submucosa rather than in cervix epithelium. Although there is scant information in the current literature regarding the presence of HPA2 within tissues as discussed earlier, there is a report of increased presence of HPA2 in cells in the peripheral blood mononuclear cell fraction in humans with breast cancer (Theodoro et al., 2007). Similarly identification of HPA2 in neurons has also been previously reported in a study investigating *HPSE2's* role in Urofacial syndrome

(OMIM#236730) (Stuart et al., 2013). There was a complete absence of labelling in lower genital tract tissues of other genotypes and disease state, additionally stage of cancer did not appear important as other samples of the same histological cancer grade, but genotypes other than 1,1 did not show labelling. The HPA2 antibody is targeted to the C terminus; therefore it is likely that the IHC study would identify isoforms one, two, three and four, but not isoform five due to its truncation, therefore identifying which isoform is being labelled is not possible. In addition to this, the multiple bands produced during amplification of the *HPSE2* isoforms suggest the presence of more than the five isoforms sequenced and it is therefore possible that one or more of these are being labelled instead.

The labelling pattern identified in our study offers further evidence that the Pv11 marker and the *HPSE2* gene are linked. Incidental labelling was also identified in the stomach of one of the UGC positive genotype 1,1 animals. HPA2 has been identified in the gastric mucosa of humans suffering from gastric neoplasia (Zhang et al., 2013) yet on reviewing the histopathology report of the animal in question, although it reported metastasis to various organs the stomach was not mentioned as one of them. One of the problems this study faced was the absence of a clear positive control tissue due to the lack of information regarding the normal presence of HPA2 both in humans and in other species. In lieu of this, tissues with a previously reported high mRNA level such as uterus and urinary bladder were considered as potential positive controls, however out of the four samples of these tissues examined labelling was not identified in any of them (including in the allele one homozygote UGC positive animals), this is not unexpected however due to the lack of correlation of mRNA expression with presence of its corresponding protein discussed previously.

HPA2 presence has been identified as a possible prognostic marker by having an inverse correlation with metastasis (Levy-Adam et al., 2010). The findings of the present study potentially imply that disease course may be slower in allele one homozygote animals. However assessing whether protein expression is associated with neoplastic spread in the CSL in this study is not possible as the majority of animals, when they are first presented, are already in an advanced state of disease and there is no

way of determining any differences in the course of disease of animals of the various genotypes.

The labelling of only a single allele type in our study strongly suggests that *HPSE2* is important in UGC in the CSL, however further investigation consisting of a larger sample set is clearly warranted. The findings of this study along with those reported previously in human studies support the need for additional research into the role of this gene.

Chapter 6

Prevalence of herpesvirus in California sea lions (*Zalophus californianus*) with urogenital carcinoma – A case-control study.

6.1 Introduction

The presence of herpesvirus intranuclear inclusion bodies in tumour tissue from a CSL was first reported approximately 14 years ago during a study investigating the primary site of UGC in CSLs (Lipscomb et al., 2000). Subsequent PCRs undertaken on DNA extracted from metastatic tissue allowed initial sequencing and phylogenetic analysis. The results of this indicated that the novel virus identified was a *gammaherpesvirus*, and placed it within the genus *Rhadinovirus* (Lipscomb et al., 2000*)*. This novel herpesvirus was subsequently named Otarine herpesvirus-1 (OtHV-1) following further work that determined it was distinct from other pinniped herpesviruses (King et al., 2002).

The *Herpesviridae* family is divided into three subfamilies, *Alpha (α), Beta (β)* and *Gamma (γ)* with OtHV-1 being in the subfamily *Gammaherpesvirinae* (King et al., 2002, Maness et al., 2011, Lipscomb et al., 2000, McGeoch et al., 2006). Herpesviruses are large DNA viruses that mainly infect vertebrate species with around 200 herpesvirus species being detected so far (Maness et al., 2011, Davison et al., 2009, McGeoch et al., 2000). In pinnipeds there are eight known herpesviruses, consisting of one α-herpesvirus (Phocid herpesvirus-1) and seven γ-herpesviruses (Phocid herpesvirus-2, Hawaiian monk seal herpesvirus, Northern elephant seal herpesvirus, Otarine herpesvirus-1, Otarine herpesvirus-2, Otarine herpesvirus-3 and Otarine herpesvirus-4 (Cortés-Hinojosa et al., 2013, Venn-Watson et al., 2012, Maness et al., 2011, Lipscomb et al., 2000, King et al., 2002, Goldstein et al., 2006b, Goldstein et al., 2006a, Osterhaus et al., 1985, King et al., 1998, Harder et al., 1996, Lebich et al., 1994)

Many herpesviruses are host species specific and evolutionary analysis of their lineage has suggested co-divergence with the host (Maness et al., 2011, McGeoch et al., 2006), however deeper analysis of mammalian γ-herpesviruses have indicated that interspecies

transfer has also played a part in their evolution (Ehlers et al., 2008). The phylogeny of OtHV-1 was established by sequencing and analysis of the DNA polymerase (Dpol) and terminase gene fragments (Lipscomb et al., 2000, King et al., 2002). Phylogenetic analysis using the Dpol gene fragment placed the newly discovered OtHV-1 close to human herpesvirus-8 (HHV-8), (Lipscomb et al., 2000, King et al., 2002). Additional research into the relationship between OtHV-1 and phocid herpes virus 2 (PHV-2), a γ-herpesvirus affecting another member of the subgroup Pinnipedia, the phocids was carried out using GCG-gap (Genetics Computer Group, global alignment) (King et al., 2002). It showed that PHV-2 was more closely related to HHV-8 and Equine herpesvirus 2 (EHV-2) and was distinct from OtHV-1. EHV-2 is postulated to play a role in immunosuppression and respiratory disease whereas so far the clinical significance of PHV-2 is unknown (Harder et al., 1996, Franchini et al., 1997, Blakeslee et al., 1975, Craig et al., 2005). Differences in the position of OtHV-1 on the phylogenetic tree were reported when comparing the analysis of the terminase fragment with that of the Dpol fragment, but both approaches identified OtHV-1 as being a γ-herpesvirus (Lipscomb et al., 2000, King et al., 2002). The herpesvirus genome contains core genes that are common to the *herpesviridae*, additionally a particular herpesvirus will contain genes that are specific to its subfamily along with genes that are found in viruses closely related to it, therefore to confirm the classification of OtHV-1 further genome sequencing is required (King et al., 2002, Alba et al., 2001).

The lifecycle of herpesviruses include both a latent and a lytic stage with the ability to establish latent infections being a hallmark of the herpesviruses (Croen, 1991, Stevens, 1989). The three subfamilies display different cell tropisms; α and β-herpesviruses establish latent infection in neurons and T cells respectively whereas the γ-herpesviruses are classed as lymphotrophic, having preference for latency within B lymphocytes (Croen, 1991). The oncogenic potential of γ-herpesviruses has been recognised in the human medical field with Epstein Barr virus (EBV, Human herpesvirus-4 (HHV-4)) of the genus lymphocryptovirus and Kaposi's sarcoma herpesvirus (KSHV, Human herpesvirus-8 (HHV-8)) of the genus rhadinovirus (Chang et al., 1994, McGeoch et al., 1995, Antman and Chang, 2000, Neipel et al., 1998) and in the veterinary field with Herpesvirus saimiri (HSV) and Herpesvirus ateles (HVA) both of the genus rhadinovirus being of interest (Melendez et al., 1970, Hunt et al., 1970, Hunt et al.,

1972, Melendez et al., 1972, McGeoch et al., 1995, McGeoch et al., 2000, Neipel et al., 1998). Infection with EBV or KSHV doesn't always result in neoplasia as it is commonly the case with viruses and cancer that the prevalence of the virus in the population is greater than the occurrence of the associated tumour (Morris et al., 1995, Monini et al., 1996). This suggests the role of other factors in cancer development. In the case of KSHV and EBV, co-infection with human immunodeficiency virus (HIV) is particularly important. The probability of developing Kaposi's sarcoma (KS) (a vascular neoplastic condition of varying severity resulting in skin lesions progressing to lesions in internal organs) is reported to increase by 60% per year following co-infection of KSHV and HIV, suggesting that duration of HIV infection is important in the pathogenesis of the disease (Jacobson et al., 2000, da Silva and de Oliveira, 2011, Antman and Chang, 2000, Patel et al., 2004). Further to this genetic susceptibility to infection with KSHV has also been recognised (Plancoulaine et al., 2003, Pedergnana et al., 2012).

EBV is associated with a number of different neoplasias in humans including B-cell lymphoma, nasopharyngeal carcinoma, Hodgkins lymphoma and the African form of Burkitts lymphoma (Zheng, 2010, Damania, 2004, Thompson and Kurzrock, 2004, Young and Rickinson, 2004). KSHV is also associated with more than one neoplastic condition alongside KS namely primary effusion lymphoma and Castleman's disease (a lymphoproliferative disorder) (Patel et al., 2004, Zheng, 2010, Soulier et al., 1995, Damania, 2004, Cai et al., 2010). HSV and HVA are oncogenic primate γ-herpesviruses that are not associated with disease in the natural reservoir host, these being the squirrel monkey (*Saimiri sciureus*) in the case of HSV and the spider monkey (*Ateles geoifroyi*) in the case of HVA (Hunt et al., 1972, Hunt et al., 1970). However experimental infection of HSV into cotton-topped marmosets (*Saguinus oedipus*) and owl monkeys (*Aotus trivirgatus*) was found to result in the development of a malignant lymphoma, this result was also seen with infection of HVA into cotton-topped marmosets (Damania, 2004, Hunt et al., 1970, Hunt et al., 1972).

It has been proposed that oncogenic transformation due to γ-herpesvirus infection is a result of the virus expressing proteins to assist its survival in the host cells (Hardie, 2010). These proteins help the virus avoid the immune system along with allowing it to

replicate during cell division (Hardie, 2010, Damania, 2004). In the case of KSHV the proteins expressed exert a variety of actions including inactivation of p53, an important cell cycle control protein frequently associated with cancer development, along with the expression of viral cytokines which encourage angiogenesis and cell proliferation (Friborg et al., 1999, Hardie, 2010, Nigro et al., 1989, Martin and Gutkind, 2009, Aoki et al., 1999). Expression of KSHV oncogenes occur in both the lytic and latent stage of the life cycle (in comparison to EBV where transformation is thought to occur only during the latent stage) (Hardie, 2010, Damania, 2004) and both genetic factors along with infection with other agents that result in immunosuppression have been implicated as co factors in assisting disease development (Damania, 2004, Jacobson et al., 2000, Plancoulaine et al., 2003, Diepstra et al., 2005).

The initial CSL tumour-herpes study was small and only tissue samples from four of the 10 animals in the study were available for the determination of herpesvirus status. However, consensus PCR identified the presence of herpesvirus DNA in all four samples and sequencing confirmed the presence of the γ-herpesvirus (Lipscomb et al., 2000). The subsequent studies carried out in 2002 and 2006 demonstrated the presence of OtHV-1 DNA by PCR in tissues from all the study animals affected by neoplasia (n=16 and 15 respectively) (King et al., 2002, Buckles et al., 2006). Of some note was that the prevalence of the virus was found to be significantly lower in non-tumour animals; only being present in tissues from two out of 17 control females and three out of eight control male animals (Buckles et al., 2006).

In animals with UGC the virus was found to be more widely disseminated in body tissues in comparison to animals in the control group, although it was found more frequently in regions affected by the tumour particularly vaginal tissue in females (78%), prostate tissue in males (80%). In addition the lumber lymph nodes were commonly affected in both sexes (60% and 78% in males and females respectively) (Buckles et al., 2006). There is the possibility that the virus is a secondary opportunistic pathogen that has taken advantage of an affected animal's malnourished state (Gulland et al., 1996, Katona and Katona-Apte, 2008). A second possibility is that latent infection with OtHV-1 is already occurring and stress placed on the cells by the presence of neoplasia drives the virus into the lytic phase. It has been shown that lytic activation of

KSHV occurs in areas of low oxygenation (Davis et al., 2001) and that the concentration of oxygen in the female urogenital tract is low and becomes even lower when inflamed (Shima et al., 2011).

Buckles et al., (2006) carried out PCRs targeting OtHV-1 Dpol fragments using DNA extracted from archived sections of non-urogenital tumours from 13 California sea lions. However they were not able to demonstrate the presence of the virus in tumour tissue from any of these animals suggesting that OtHV-1 may not be an opportunistic virus. If it were it might also be expected in these cases, secondary herpesviral infections have been seen to occur in immunosuppressed cancer patients (Wong and Hirsch, 1984). The 13 animals consisted of three juveniles and 10 adults of both sexes with varying type and site of tumour, five out of the eight of the tumour types were malignant. This study also found the virus was absent in juvenile animals.

A further study investigated the prevalence of the virus in pharyngeal and urogenital tract swabs and in peripheral blood mononuclear cells (PBMC) from 212 wild caught animals at two study sites (San Miguel Island, California and Puget Sound, Washington), of which 112 were immature along with 27 adults and 12 juveniles admitted to TMMC (Buckles et al., 2007). Detection of virus from pharyngeal swabs was low with a prevalence of only 2% and the virus was undetected in PBMC, urogenital tract swabs however showed a higher detection rate. OtHV-1 was identified by PCR in the immature animals, but its prevalence was at a significantly lower level than in the adults (5.8% compared to 19.6% ($p<0.05$) and 44.1% ($p<0.05$) in adult females and males respectively). The decreased presence of virus in immature animals along with a higher detection rates in urogenital secretions points towards OtHV-1 being a sexually transmitted infection. The study drew a parallel between the epidemiology of OtHV-1 infection in CSL with infection of humans with the closely related KSHV (HHV-8) (Buckles et al., 2007). Studies on the epidemiology of KSHV have suggested that sexual activity plays an important role in the transmission of the virus (Monini et al., 1996) particularly in the case of AIDS associated KS, where the existence of another co-factor is suspected (Kedes et al., 1996, Cai et al., 2010). However a definitive answer regarding transmission of KSHV is yet to be found as transmission appears to vary according to endemicity. Non-endemic areas appear to

have a high level of spread by sexual contact whereas in endemic areas sexual contact, vertical transmission and transmission between siblings is also of importance (Cai et al., 2010, Plancoulaine et al., 2000, Hengge et al., 2002, Lacoste et al., 2000, Martin et al., 1998, de-The et al., 1999).

In the study carried out by Buckles et al, (2007) the prevalence of OtHV-1 in adult males was significantly higher than in adult females ($p<0.05$). At present the reason for this is unknown but it is postulated that if OtHV-1 is sexually transmitted a number of factors may play a part. Differences in the microenvironment of the genital tract of males and females could influence success of infection, along with the polygynous mating behaviour of CSL that potentially results in more males contracting the virus through a higher number of sexual contacts (Buckles et al., 2007). However as with the closely related virus KSHV, the virus associated with KS in humans other routes of transmission should not be ruled out as sexually immature animals have been identified with the virus albeit at a much lower prevalence (Buckles et al., 2007).

The present study further investigates the potential involvement of herpesvirus in cases of UGC among CSL. Previous studies have produced contradictory results with a relatively high occurrence of the virus identified in presumably healthy adult wild animals (Buckles et al., 2007), but a low level found in stranded neoplasia negative animals along with animals with tumours other than UGC and a high occurrence in adults with neoplasia (Buckles et al., 2006, King et al., 2002). However it should be remembered that it is not known how many of the wild caught animals sampled in the study by Buckles et al., (2007) went on to develop UGC. The sampling technique also varied between the studies, with the initial work isolating viral DNA from tissues at necropsy (Buckles et al., 2006, King et al., 2002) and the later study isolating it from swabs (Buckles et al., 2007). In cases of UGC detection of the virus was higher in samples from the urogenital tract and sub-lumber lymph nodes than in the other body tissues (Buckles et al., 2006).

In comparison to previous studies investigating the involvement of OtHV-1 in UGC (King et al., 2002, Lipscomb et al., 2000, Buckles et al., 2007), the present study only included DNA from lower genital tract tissue from adult female animals thereby removing sex and age class as confounding variables. This study was designed in a

case-control fashion. It included a larger sample size, thereby increasing the power of the study, than previous work. Furthermore as detailed in Chapter 7 the study was able to explore potential co-factors involved in UGC as a result of additional information available in necropsy reports for each animal entered into the study.

The aims of the study were to (i) determine the prevalence of herpesvirus in the CSL using a pan-herpes screening PCR that employed degenerate primers targeted to the conserved DNA polymerase gene (Dpol). The pan-herpes PCR would potentially enable the detection of more than one herpesvirus species in the CSL in the study. (ii) To establish whether OtHV-1 specifically is associated with the occurrence of UGC in the CSL again using a PCR targeting the Dpol gene. In both instances DNA extracted from lower genital tract tissues was used as a template due to previous investigations that identified that OtHV-1 is more frequently found in urogenital tract tissue (Buckles et al., 2006). (iii) Using Pv11 genotype data obtained earlier in this study to investigate whether there was an association between herpesvirus infection and homozygosity at the Pv11 locus.

6.2 Materials and Methods

6.2.1. Preparation for herpesvirus PCR

Sample collection

Tissue samples from the lower genital tract were collected from 65 female adult animals during necropsy examinations carried out by staff at the Marine Mammal Center, Sausalito, CA, USA. These consisted of 54 cervical tissue samples along with nine cervix/vagina samples and two proximal vaginal samples, 64 of the samples were from animals sampled in Chapter 2. The gross necropsy and histopathology reports were reviewed and the animals classified according to cause of death. UGC was diagnosed in 23 animals and 42 were considered as control animals; having died or being euthanased due to a condition other than UGC as detailed in Figure 6.1.

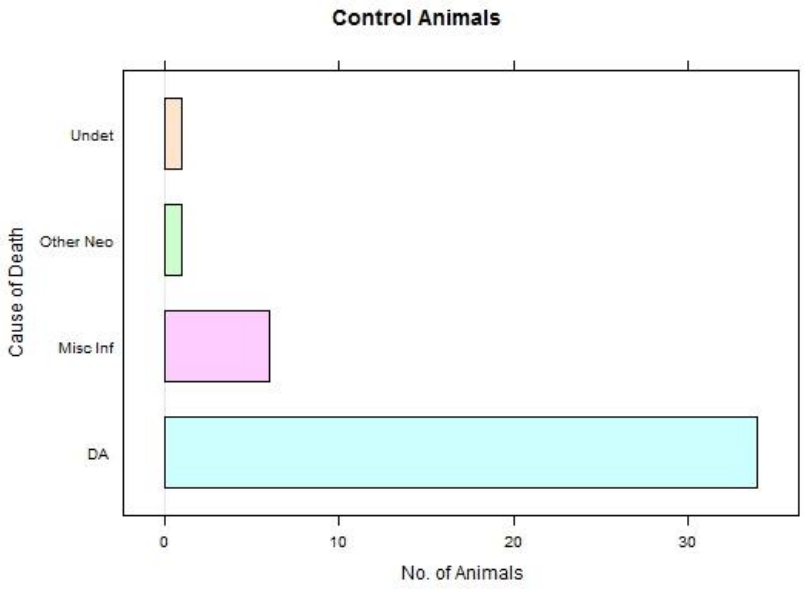


Fig 6.1. Cause of death of control animals based on predominant necropsy finding recorded. (Undet: Undetermined, Other Neo: Neoplasia other than UGC identified; Misc Inf: Miscellaneous Infection; DA: Domoic acid). Undetermined indicates cases where a cause of death was not determined, however urogenital carcinoma (UGC) was not identified.

Tissues were shipped on dry ice to the Sea Mammal Research Unit, St Andrews, Fife where they were stored at -80°C prior to use.

DNA extraction and preparation

DNA extraction and quantification was carried out as detailed in Chapter 4 (page 57). Following quantification samples were diluted with MilliQ water to a concentration of 50 ng/µl. The samples were then stored at -20°C prior to use.

6.2.2 Amplification of a DNA polymerase gene fragment of herpesvirus

The Dpol gene is involved in DNA replication and is present in a number of viruses including herpesvirus (Joyce and Steitz, 1994, Earl et al., 1986). It has been identified as a conserved core gene in the genome and therefore is a useful target in investigating herpesvirus presence (Alba et al., 2001, VanDevanter et al., 1996, Ito and Braithwaite, 1991). The two different PCRs carried out in this study used primers that amplified across different fragments of the Dpol gene.

6.2.2.1 Pan-herpes PCR

It is common for animals to be infected with more than one herpesvirus (Prepens et al., 2007) and in order to investigate the presence of other herpesviruses in the lower genital tract tissues a pan-herpes screening protocol was carried out. The protocol, involved a nested PCR that was not specific to any one herpesvirus species but instead could identify the presence of many herpesviruses (Ehlers et al., 1999, VanDevanter et al., 1996). It employed degenerate primers due to their ability to identify a number of species of herpesvirus by targeting highly conserved regions within the Dpol gene without requiring the exact sequence of each virus to be known (VanDevanter et al., 1996). The degenerate primers were further modified by replacing positions that had three or four fold degeneration with a deoxyinosine base, as it was reported previously to increase product yield (Ehlers et al., 1999). The primer sequences are detailed in Tables 6.1 and 6.2.

Table 6.1 Primer sequences for primary pan-herpes PCR

Primer	Sequence 5'-3'
Forward	
DFA*	GAY TTY GCI AGY YTI TAY CC
ILK*	TCC TGG ACA AGC AGC ARI YSG CIM TIA A
Reverse	
KGI*	GTC TTG CTC ACC AGI TCI ACI CCY TT

*(Ehlers et al., 1999) I=deoxyinosine substitution

Table 6.2 Primer sequences for secondary pan-herpes PCR

Primer	Sequence 5'-3'
Forward	
TGV*	TGT AAC TCG GTG TAY GGI TTY ACI GGI GT
Reverse	
IYG*	CAC AGA GTC CGT RTC ICC RTA IAT

(Ehlers et al., 1999) I=deoxyinosine substitution

The pan herpes screening protocol involved a nested PCR. Both reactions used Qiagen HotStarTaq (Qiagen, Crawley, UK) with a reaction volume of 50 µl. The primers were obtained from Invitrogen (Paisley, UK). The reaction mix and cycling conditions were as per Madeleine Maley, University of Edinburgh (personal communication).

The 50 µl reaction mix for the primary PCR contained per reaction; 5 µl 10×Buffer (Qiagen, Crawley, UK), 5 µl of 10 µM primer DFA (forward), 5 µl of 10 µM primer ILK (forward), 5 µl of 10 µM primer KGI (reverse), 1 µl of 10 mM dNTPs (Qiagen, Crawley, UK), 0.25 µl (1.25 units) HotStarTaq (Qiagen, Crawley, UK), 20.75 µl MilliQ water and 8 µl of DNA template (400 ng). The 50 µl nested reaction used 1 µl of the product of the primary reaction diluted 1:5 with MilliQ water (Qiagen, Crawley, UK) as a template (the template was not quantified prior to the nested reaction), the reaction mix was made up to 50 µl with 5 µl 10×Buffer (Qiagen, Crawley, UK), 5µl of 10 µM TGV primer (forward), 5 µl of 10 µM IYG (reverse) primer, 1 µl of 10 mM dNTPS (Qiagen, Crawley, UK), 0.25 µl (1.25 units) HotStarTaq (Qiagen, Crawley, UK) and 32.75 µl MilliQ water Negative controls were included in both the primary and the nested PCRs by substituting the DNA template with the same volume of MilliQ water.

The cycle conditions for both PCR reactions were as follows using a PTC-200 DNA Engine Cycler (Bio-Rad Laboratories Inc., Hercules, USA) 95°C for 15 min, followed by 45 cycles of 94°C for 30 s, 46°C for 60 s, 72°C for 60 s, then 72°C for 10 min before being held at 4°C.

The primary PCR reaction had an expected product size of approximately 440 bp. The expected product size of the nested PCR was approximately 220 bp. Only the products of the nested PCR reaction were evaluated by gel electrophoresis and to do this 5 µl of PCR product mixed with 2.5 µl Orange G loading dye (Sigma-Aldrich Ltd, Gillingham, UK) was then resolved at 80V for 30 min on a 1.5% agarose gel containing 2.5 µl ethidium bromide (Sigma-Aldrich Ltd, Gillingham, UK) alongside 2.5 µl of 100 bp size standard (GeneRuler^{TM} 100 bp Plus DNA Ladder, Thermo Fisher Scientific, Loughborough, UK). Bands were then visualised in a UV light box (UVITEC, Cambridge, UK). PCRs were repeated on 50% of the samples that were found to be negative on gel electrophoresis to confirm the result.

6.2.2.2 Otarine herpesvirus -1 PCR

The OtHV-1 specific PCR used primers that were specifically targeted to a fragment of the Dpol gene in the OtHV-1, the primers were obtained from Invitrogen (Paisley, UK) and the sequences are detailed in Table 6.3. The reaction mix and cycling conditions were as per Dr Tracey Goldstein, UC Davis Wildlife Health Center, California, USA (personal communication).

The 25 µl reaction mix consisted of 2.5 µl 10 x PCR Buffer (Invitrogen, Paisley, UK), 0.75 µl of 50mM $MgCl_2$ (Invitrogen, Paisley, UK) (50 mM), 0.5 µl PolFor (10 µM forward primer), 0.5 µl PolRev (10 µM reverse primer), 0.5 µl of 10 mM dNTPs (Qiagen, Crawley, UK), 0.1 µl (0.5 unit) Platinum® *Taq* DNA polymerase (Invitrogen, Paisley, UK), 5 µl DNA template (250 ng) and 15.15 µl MilliQ water. The cycle conditions for the PCR reaction was as follows using a PTC-200 DNA Engine Cycler (Bio-Rad Laboratories Inc., Hercules, USA) 94°C for 2 min, followed by 35 cycles of 94°C for 40 s, 54°C for 40 s, 72°C for 40 s, then 72°C for 10 min before being held at 4°C. A negative control was included by substituting the DNA template with an equal volume of MilliQ water.

The PCR reaction had an expected product size of approximately 740 bp. To analyse the fragments 5 µl of PCR product was mixed with 2.5 µl Orange G loading dye (Sigma-Aldrich Ltd, Gillingham, UK) and resolved at 80V for 30 min on a 1.5% agarose gel containing 2.5 µl ethidium bromide (Sigma-Aldrich, Gillingham, UK) alongside 2.5 µl of 1kb size standard (1Kb Ladder, Invitrogen, Paisley, UK). The presence of bands of an appropriate size were identified by visualisation in a UV light box (UVITEC, Cambridge, UK). PCRs were repeated on negative samples to confirm the result.

Table 6.3 Primer sequences for OtHV-1 specific PCR

Primer	Sequence 5'-3'	Reference
PolFor	TTA CAC TTC TAC GTG ATG G	(Buckles et al., 2007)
PolRev*	TCT TCG TCC AGT ATC ATT G	(Buckles et al., 2007)

*The reverse primer in the paper referenced (Buckles et al., 2007) is published in the wrong direction, the correct 5'-3' sequence is shown here.

The position of the primers used in both the pan herpes PCR and the OtHV-1 specific PCR are detailed in Figure 6.2. The OtHV-1 gene fragment (NCBI GenBank: AF236050.1) was aligned with the DNA polymerase gene from Human Herpesvirus-4 (NCBI GenBank: NC_007605.1) in order to illustrate the position of all of the primer sites.

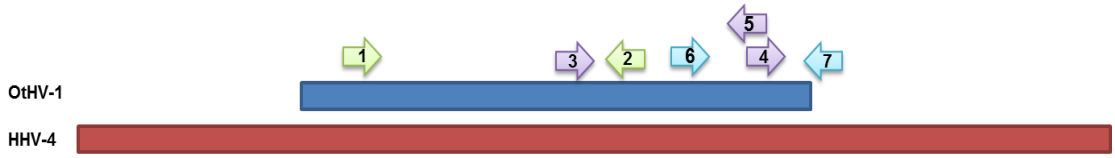


Fig 6.2. Illustration of the location of primers (indicated by the arrows) used in the two PCR protocols to detect the presence of herpesvirus in UGT of CSL. Green arrows indicate the sites of the primers used in the OtHV-1 specific PCR, purple arrows indicate the sites of the primers used in the primary pan-herpes PCR and the blue arrows indicate the sites of the primers used in the secondary pan-herpes PCR. The whole of the DNA polymerase gene of human herpesvirus 4 (HHV-4) is aligned with the known fragment of the Otarine herpesvirus-1 (OtHV-1) gene in order to indicated all the sites of the primers used in the pan-herpes protocol. Arrow 1] Forward primer (PolFor) for OtHV-1 specific PCR; 2] Reverse primer (PolRev) for OtHV-1 specific PCR; 3] Pan-herpes primary PCR forward primer DFA; 4] Pan-herpes primary PCR forward primer ILK; 5] Pan-herpes primary PCR reverse primer KGI; 6] Pan-herpes secondary PCR forward primer TGV; 7] Pan-herpes secondary PCR reverse primer IYG.

6.2.2.3 Analysis of PCR products

In the case of both the pan-herpes PCR and the OtHV-1 specific PCR, products deemed positive on agarose gel were purified using MSB Spin PCRapace PCR purification kit (Stratec molecular, Berlin, Germany) and 5 µl of the purified product mixed with 2.5 µl Orange G loading dye (Sigma-Aldrich Ltd, Gillingham, UK) and resolved on 1% agarose gel containing 2.5 µl 10mg/ml ethidium bromide (Sigma-Aldrich Ltd, Gillingham, UK), alongside three lambda DNA size markers; 25ng, 50ng and 100ng (Promega, Southampton, UK) for quantification. In the case of the pan-herpes screen, where possible, 20 ng of PCR product was submitted for sequencing along with 3.2 pmol of forward or reverse primer (TGV and IYG). For the OtHV-1 specific PCR, where possible 40 ng of PCR product was submitted for sequencing along with 3.2 pmol of forward or reverse primer (PolFor and PolRev). All of the pan-herpes PCR samples and OtHV-1 samples seen to be positive on gel electrophoresis were submitted for sequencing. DNA sequencing was performed by DNA Sequencing & Services

(MRCPPU, College of Life Sciences, University of Dundee, Scotland, www.dnaseq.co.uk) using Applied Biosystems Big-Dye Ver 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer.

DNA sequence analysis undertaken on sequences obtained from both PCRs were achieved via multiple alignment, following removal of primer sequences and performed using the software programme Geneious Pro v5.6.6 created by Biomatters. Available from http://www.geneious.com/). In addition the multiple alignment of sequences obtained from the pan-herpes PCR was undertaken with four pinniped γ-herpesviruses from the NCBI GenBank database and included partial CDS of the Dpol gene from OtHV-1; AF236050, Phocid herpesvirus-2; GQ429152, Hawaiian monk seal herpesvirus; DQ093191 and Northern elephant seal herpesvirus; DQ183057 (King et al., 2002, Maness et al., 2011, Goldstein et al., 2006a, Goldstein et al., 2006b). For sequences obtained via the OtHV-1 specific PCR the multiple alignment was carried out with the partial CDS of the Dpol gene from OtHV-1(GenBank: AF236050) (King et al., 2002).

6.2.3 Statistical analysis

Statistical significance with regards to cause of death and presence of herpesvirus from both the results of the pan-herpes screen and OtHV-1 specific PCR screen was carried out using fisher exact tests. In addition, genetic susceptibility at the Pv11 locus to infection was investigated using the genotype data established in Chapter 2, fishers exact tests were carried out using the results of both PCRs to identify any association with homozygosity at this locus and presence of herpesvirus. Statistical analysis was carried out using the open access statistical software package R (R Development Core Team, 2012). In all tests a p-value of <0.05 was considered statistically significant.

6.2.4 Problems encountered

A number of problems were encountered while undertaking this work:

Pan herpes PCR: The herpes status of the animals was unknown which meant the absence of a positive control. The PCR initially produced no positive results, however

this was later found to be the result of a defective thermocycler. On repeating the reactions on a different machine positive results were seen. Contamination was additionally an issue, this was resolved by both preparing the PCR mix in a DNA free area, but also by irradiating the pipettes and tubes with UV light for approximately 10 min prior to use. Further to this the identification of reactions not affected by contamination was difficult due to the nested PCR reaction resulting in a band around the expected product size even in the absence of DNA template. This issue was confirmed by carrying out the PCR three times without template DNA and including in each nested reaction either 1] the IYG primer only, 2] the TGV primer only or 3] both primers. When both primers were included a band was identified at ~200bp (Figure 6.3). This artefact was not a consistent finding in all the PCRs run, however any bands appearing in the negative control were sent for sequencing to rule out true contamination.

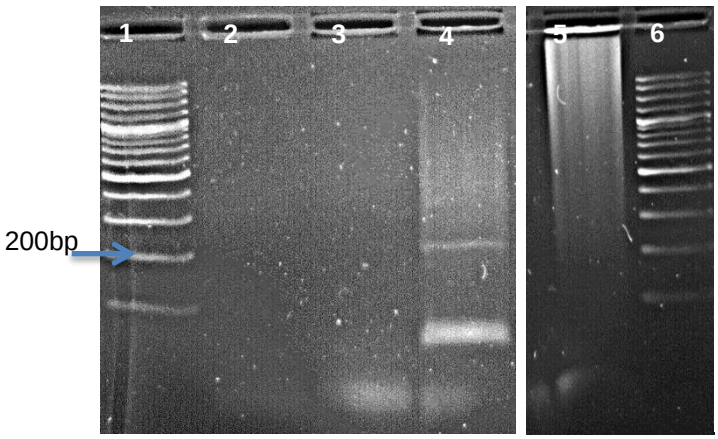


Fig 6.3. Gel electrophoresis of PCR products without DNA template to investigate primer artefacts. Lane 1] 100bp ladder, 2] PCR mix (no primers), 3] Primer TGV only, 4] Primers TGV and IYG, 5] Primer IYG only, 6] 100bp ladder. Lane 4 clearly shows a band where the expected product size for the pan-herpes PCR would be.

OtHV-1 PCR: Undertaking the pan herpes PCR screen allowed the identification of potential positive controls for the OtHV-1 PCR, however the OtHV-1 PCR initially did not result in any positive samples. A gradient PCR was carried out to identify whether the provided protocol was compatible with the thermocycler available. The original protocol stated an annealing temperature of 63°C, however the gradient PCR (run with annealing temperature between 53°C and 65°C) gave a positive result at 53°C and 55°C only (Figure 6.4) therefore the annealing temperature was reduced to 54°C for the subsequent reactions. Issues with contamination were resolved as above.

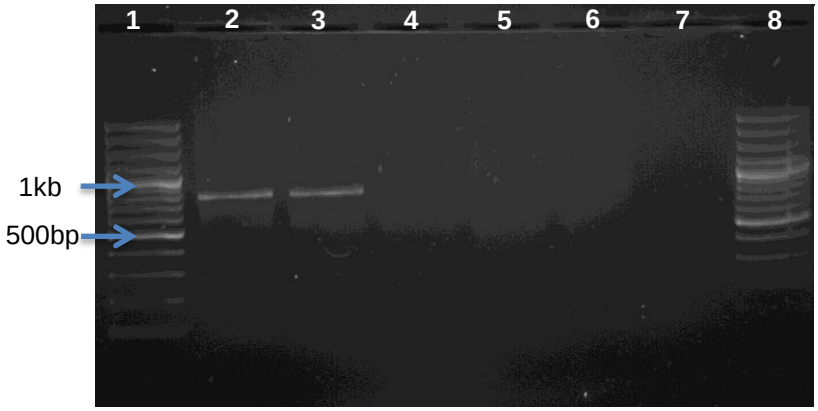


Fig 6.4. Gel electrophoresis of OtHV-1 specific PCR using sample 9770(42) at a gradient. Lane 1] 1kb ladder, 2] 53°C, 3] 55.1°C, 4] 58.6°C, 5] 62.6°C, 6] 65°C, 7] Negative control (MilliQ H_2O). Annealing temperature 53°C and 55.1°C show a positive result.

6.3 Results

6.3.1 Pan-herpes PCR

On examination of gels following gel electrophoresis of PCR products a band of appropriate size was identified in 38 samples and all 38 were submitted for sequencing. The repeated PCRs on 50% of the negative samples again resulted in negative results. An example of a positive gel is shown in Figure 6.5. Sequencing was performed in both directions and sequences of adequate quality from both forward and reverse primers were obtained for 24 of the samples. Only forward or reverse reads were obtained for eight of the samples and the remaining six samples were of inadequate quality for further analysis (consisting of samples from one UGC positive animal and five controls) and were therefore removed from the study. Removal of primer sequences and trimming of ends to remove bases of inadequate quality was carried out on the reads from the 32 samples prior to multiple alignment and resulted in sequence reads between 83bp to 166bp. On sequence analysis sequences were found to be identical and all of the samples were identified as OtHV-1.

Out of the 59 samples where herpesvirus status was determined, 32 were identified as positive for herpesvirus, giving an overall prevalence of 54%. Out of the 22 animals diagnosed with UGC 11 were confirmed positive for OtHV-1 giving a prevalence of 50 % in affected animals, whereas in control animals the prevalence was found to be 57%.

Statistical analysis did not find herpesvirus presence significantly associated with cancer; p=0.788, OR (crude): 0.77 (95% CI: 0.23-2.50).

Homozygosity at the Pv11 locus was not found to be significantly associated with the presence of herpesvirus; p=1.00, OR (crude): 1.03 (95% CI: 0.32-3.26).

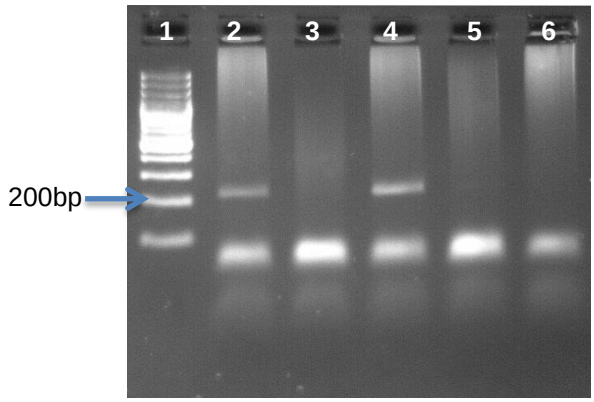


Fig 6.5. Example of a gel following pan-herpes PCR illustrating two positive samples. Lane 1] 100bp ladder, 2] 9827(41), 3] 8431(69), 4] 9770(42), 5] 9205(19), 6] Negative control

6.3.2 OtHV-1 PCR

All 16 samples deemed positive on gel electrophoresis were submitted for sequencing in both directions. The repeated PCRs on samples found to be negative again yielded negative results. An example of a gel with bands of expected size is illustrated in Figure 6.6. Sequences obtained from two samples (consisting of one UGC positive and one control) were deemed of inadequate quality and therefore removed from the analysis. Sequences from the remaining 14 samples were trimmed to remove bases of inadequate quality resulting in sequence reads of between 396bp and 688bp. A multiple alignment was carried out with all the sequences aligning with OtHV-1 (GenBank: AF236050) (King et al., 2002) as expected.

Out of the 63 samples where OtHV-1 status was determined, 14 were found to be positive for OtHV-1 giving an overall prevalence of 22%. Out of the 22 animals diagnosed with UGC 11 were positive for OtHV-1 giving a prevalence of 50% in affected animals, whereas in control animals the prevalence was found to be 7%.

Statistical analysis identified a strongly significant relationship between the occurrence of UGC and the presence of OtHV-1; p=0.0002, OR (crude): 12.04 (95% CI: 2.59-79.11). Homozygosity at the Pv11 locus was again not found to be significantly

associated with the presence of OtHV-1; p= 0.364, OR (crude): 0.52 (95% CI: 0.12-2.02).

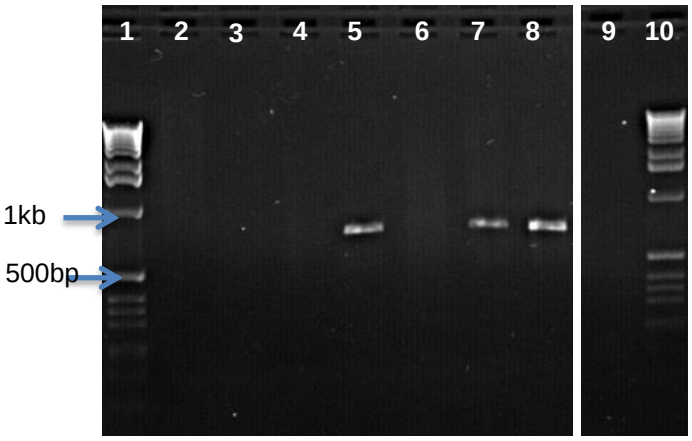


Fig 6.6. Example of a gel following OtHV-1 PCR illustrating three positive samples. Lane 1] 1kb ladder, 2] 9100(77), 3] 9196(78), 4] 8958(79), 5] 9008(80), 6] 9804(81), 7] 7140(82), 8] 7468(83), 9] Negative control, 10] 1kb ladder

6.4 Discussion

The results from the two PCRs give extremely different outcomes, with the pan-herpes PCR not identifying a significant association between the presence of herpesvirus and UGC and the OtHV-1 specific PCR resulting in a highly significant relationship. An association was not identified from the results of either PCR between the presence of herpesvirus and homozygosity at the Pv11 locus.

The pan-herpes PCR, identified a single virus on sequencing; OtHV-1. The prevalence of which in the animals in the study was found to be 54%, this is in comparison to 22% of OtHV-1 prevalence identified using the OtHV-1 specific PCR. The higher prevalence identified by the pan-herpes screen could be explained by the increased number of amplification cycles as a result of the nested protocol thereby increasing its sensitivity (90 across the two PCRs in comparison to the 35 cycles in the OtHV-1 specific PCR). Quantitative PCR has been recognised as more sensitive than the standard OtHV-1 protocol (Buckles et al., 2007), therefore implementing this protocol in future may assist in determining if a sample is truly negative.

Alternatively, as the primers used in the two PCRs amplify across different fragments of the Dpol gene there is the potential that variation in the sequence is occurring, but not

within the region amplified by the degenerate primers therefore the PCR is detecting a closely related virus or a variant of the OtHV-1 virus. Variants of a herpesvirus have been identified in other vertebrates. Elephants, in particular Asian elephants (*Elephas maximus*) are affected by an acute haemorrhagic disease which has been associated with infection with β-herpesviruses of a new genus; *Proboscivirus,* known as Elephant Endotheliotropic Herpesviruses (EEHV) (Richman et al., 1999, Latimer et al., 2011, Ehlers et al., 2001, McGeoch et al., 2006). One of which; EEHV1 was identified as having two variants (EEHV1A and EEHV1B) following sequence analysis of the terminase gene (Latimer et al., 2011, Fickel et al., 2001).

It is expected that the pan-herpes PCR would identify all the herpesvirus positive animals in the study, however six samples found to be positive in the OtHV-1 specific PCR were negative in the pan-herpes screen. Pan-herpes PCRs were repeated on four of these samples with the results again being negative, therefore implying that false negatives occur with this protocol. An observation supporting this theory was made in a study again investigating elephant herpesviruses where it was noted that sequence variation was occurring in the primer binding sites of the pan-herpes primers used, resulting in reduced detection of virus compared with a virus specific PCR (Latimer et al., 2011).

It should also be remembered that it is common for vertebrates to harbour more than one herpesvirus and during pan-herpes PCR screening the dominant virus present is more likely to be amplified (Ehlers et al., 2008, Prepens et al., 2007) therefore the presence of other herpesvirus species in the tissues examined cannot be ruled out. It has been suggested that the identification of different herpesvirus within a sample can be undertaken by degenerate PCR targeting the glycoprotein B (gB) gene alongside the pan-herpes PCR targeting the Dpol gene. This approach has been seen to successfully identify more than one herpesvirus in blood samples from primates including Black-and-White Colobus Monkeys (*Colobus guereza*) and cynomolgus monkeys (*Macaca fascicularis*) (Prepens et al., 2007, Ehlers et al., 2008).

In spite of these complications the prevalence of 50% identified in lower genital tract tissues of affected animals by both PCR protocols is comparable to the previous finding of 55% in cervix tissue from animals with UGC (Buckles et al., 2006). However the

present study did identify a higher level of virus in tissue from the lower genital tract of control animals. The pan-herpes PCR and OtHV-1 PCR gave prevalence's of 57% and 7% respectively, unlike the previous study where it was undetected in lower genital tract tissues from female control animals (Buckles et al., 2006). This may be a result of the larger sample size or changing prevalence of the virus in the population over time. Further to this in the case of the pan-herpes protocol, the potential additional detection of a closely related virus as mentioned above may contribute to higher detection; even so the high prevalence in non-cancer animals suggests that OtHV-1 (or a closely related virus or variant) is widespread in the population.

The life cycle stage of the herpesvirus may also influence whether detection occurs. The viral DNA load in tissues increases when cells enter the lytic stage of their life cycle as a result of virus reactivation and replication, therefore detection of viral DNA via PCR during this stage is more likely (Pusterla et al., 2009, Lunn et al., 2009, Traylen et al., 2011). It was noted that detection of EEHV by PCR using primers targeting the gB gene in latent infection was not reliable as a potential screening technique as the virus was only detected in samples (tissue and blood) from affected animals and not suspected carriers (Fickel et al., 2003). Therefore although the results of the present study suggest a highly significant relationship between the presence of OtHV-1 and UGC in CSL this may be more a result of lack of detection in carrier animals rather than the increased presence in UGC positive animals. This would also suggest that the viral load is higher in lower genital tract tissues of UGC positive animals potentially due to the virus being placed under stress and entering the lytic stage, whereas in animals unaffected by UGC if OtHV-1 is present it is more likely to be latent in the lower genital tract tissues.

The association of an infectious agent in the aetiology of UGC in CSL has potential implications for captive management of these animals, if the maintenance of a disease free population of CSL is desired. Additionally OtHV-1 has been recently identified in another member of the Otariidae family, a South American fur seal (*Arctocephalus australis*) suffering from UGC in a UK zoo (Dagleish et al., 2013). In light of this finding it was proposed that screening of otrarids for OtHV-1 should be considered prior to relocating an animal to another institution (Dagleish et al., 2013). The success

of this however would depend on the availability of a test with enough sensitivity to detect latent infection.

Although there is a clear need for further investigation into the involvement of herpesvirus in UGC in CSLs, this study supports previous work and provides a strong indication that the presence of OtHV-1 is a risk factor for the disease.

Chapter 7

Risk factors associated with the development of urogenital carcinoma in the California Sea Lion (*Zalophus californianus*)

7.1 Introduction

The multi-factorial aetiology of neoplasia presents great difficulties in establishing the cause of the disease and necessitates the investigation of a number of factors. These challenges are amplified in wildlife studies, where achieving an adequate sample size, in order to investigate suspected risk factors is particularly problematic (Newman and Smith, 2006, McAloose and Newton, 2009). The present study has made use of archived skin and lower genital tract tissue samples stored at the Marine Mammal Center, enabling the opportunity to include a larger sample size than is frequently possible. In addition to the tissues samples from each of the 113 animals entered into the study, necropsy reports consisting of gross pathology and histopathology were available. These reports contained body condition data detailing body length, mass, girth and blubber thickness, thereby providing further information about each animal that could be analysed alongside the Pv11 genotype and herpesvirus status determined by this study.

This work has so far demonstrated an association both with *HPSE2* (Pv11) genotype and OtHV-1 status and the presence of UGC in the CSL. Animals identified as homozygous at the Pv11 microsatellite within the *HPSE2* gene were identified as almost twice as likely to have cancer. Further to this the identification of differential labelling of only one homozygous genotype in tissues from animals with UGC supports the notion that the Pv11 marker and the *HPSE2* gene are linked. PCRs undertaken on DNA extracted from lower genital tract tissues targeting the Dpol gene of the OtHV-1 virus found that the presence of the virus was significantly associated with UGC. The aim of this final chapter is to bring together the findings of the previous chapters along with an analysis using the additional body condition data.

7.2 Materials and Methods

Two binomial generalised linear models (GLMs) were fitted to the data. The first involved all 113 animals (43 cases and 70 controls) and investigated the influence of the following variables on the presence of UGC; previously determined homozygous or heterozygous genotype of Pv11, M11a and Hg8.10 (Chapter 2 and appendix A), along with body length, mass, girth and ventral blubber thickness as provided in the necropsy reports.

The second GLM involved the 57 animals (21 cases and 36 controls) where lower genital tract tissue was also available. The study additionally included data on herpesvirus status in order to investigate the effect that the presence of herpesvirus may have on the likelihood of cancer. Genotype and herpesvirus status was previously determined as detailed in Chapters 2, 6 and appendix B and body condition data was supplied in the necropsy reports.

The GLMs were carried out using the open access statistical software package R (R Development Core Team, 2012).

7.3 Results

The first GLM consisting of the 113 animals investigated all variables. The "step" function was then applied to the model to identify the variables providing the best fit.

The best model included the variables Pv11 genotype, Blubber thickness, mass and girth (with an Akaike's Information Criterion (AIC) of 142.6). A significant relationship was identified between presence of cancer, homozygous Pv11 genotype ($p= 0.02950$) and blubber thickness ($p= 0.00381$) and although the other variables were not individually significant this model selection (stepwise) process indicated they should remain in the model.

However, linear models and pairwise plots (Figure 7.1) identified correlations between blubber thickness, girth and body mass. Thus including all of them in the model would violate the assumption of GLMs that the independent variables are not related to each

other. The GLM was repeated including either blubber thickness, girth or body mass (alongside Pv11 genotype) giving AIC scores of 141.7, 153.2 and 151.1 respectively. The lower AIC score identified with blubber thickness in the model suggested that its inclusion out of the three body condition measures provides the best fit. Therefore girth and mass were removed from the model and the GLM repeated investigating the effect of only Pv11 genotype and blubber thickness on the presence of cancer.

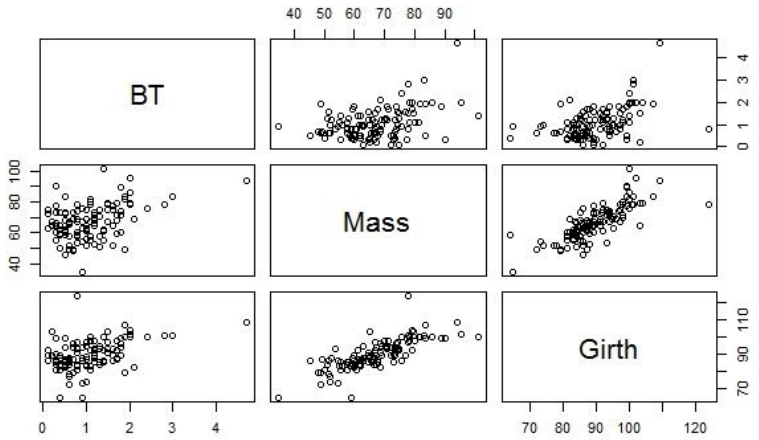


Fig. 7.1 Pairwise plots investigating correlations between three body condition variables; blubber thickness (BT), body, mass and girth. Correlations are seen between all three.

The results of the model again identified a significant relationship between the presence of cancer and homozygosity at the Pv11 locus and also between the presence of cancer and thinner blubber (Table 7.1). The difference between blubber thickness in UGC positive and control animals for the 113 animals is illustrated in Figure 2.

Table 7.1 GLM results showing a significant relationship ($p<0.05$) between homozygosity at the Pv11 locus and blubber thickness with the presence of UGC. 113 animals were included in the model.

Variable	Estimate	Standard error	Pr(>|z|)
***Pv11GTHO**	0.9474	0.4633	0.04087
****BT**	-1.1842	0.3905	0.00243

*Pv11 homozygous genotype; **Blubber thickness

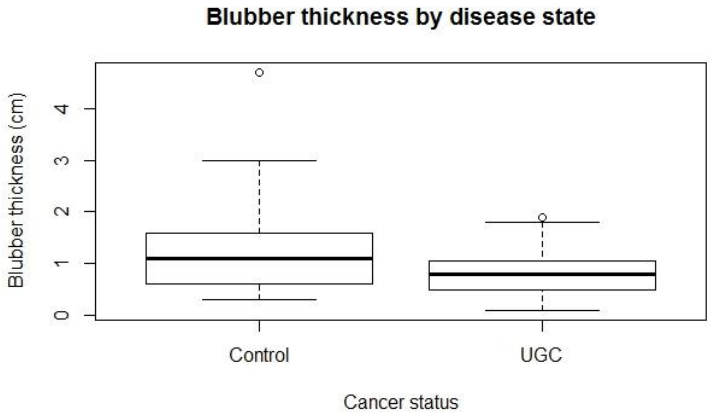


Fig 7.2. Comparison of blubber thickness in 113 CSLs; 70 controls and 43 with UGC. Animals with UGC have significantly thinner blubber.

The adjusted odds ratio for the association of homozygosity at the Pv11 locus was also recorded with UGC found to be over twice as likely in animals homozygous at the Pv11 locus (Table 7.2).

Table 7.2 Crude and adjusted odds ratios for Pv11 genotype showing that UGC is over twice as likely in animals homozygous at the Pv11 locus. Significance accepted at the 5% level.

Variable	Crude OR (95% CI)	Adjusted OR (95% CI)	P(LR-test)
***Pv11GT: HO vs HT**	2 (0.87,4.6)	2.58 (1.04,6.39)	0.039

*Pvll genotype, HO: homozygous; HT: heterozygous

The second model included fewer animals (57) as lower genital tract tissues used to investigate herpesvirus status were only available for a subset of the animals. In addition eight animals were removed due to an inconclusive herpesvirus PCR result.

The variables included in the model were Pv11, M11a and Hg8.10 genotype, pan-herpes virus PCR results (positive or negative) and OtHV-1 specific PCR results (positive or negative) and blubber thickness. Due to the correlation identified between the body condition measures (Figure 7.1), only one body condition measure was included in the model. Blubber thickness was chosen due to its previously identified importance in predicting cancer status. Further to this it is not affected by other pathological processes which could potentially affect the other measures, an example being an increase in body mass due to ascites or organomegaly which would make an animal appear in better condition than it actually was.

The "step" function was again used and best model identified was found to include blubber thickness, OtHV-1 status specifically and Pv11 genotype (AIC 60.73). The results of this GLM are shown in Table 7.3. This model identified a significant relationship between the presence of cancer and homozygosity at the Pv11 locus and positive OtHV-1 status, but unlike the model consisting of 113 animals a significant relationship was not identified regarding blubber thickness.

Table 7.3 GLM results showing a significant relationship ($p<0.05$) between homozygosity at the Pv11 locus and OtHV-1 postive virus status with the presence of UGC. 57 animals were included in the model.

Variable	Estimate	Standard Error	Pr(>|z|)
***Pv11GTHO**	1.8732	0.7032	0.00772
****BT**	-0.9382	0.5942	0.11438
*****OtHV-1Pos**	2.3141	0.8431	0.00606

*Pv11 homozygous genotype; **Blubber thickness;***OtHV-1 status as gained from the OtHV-1 specific PCR

Odds ratios were also recorded for Pv11 genotype and OtHV-1 status as detailed in Table 7.4. The odds ratios recorded were much greater than in the previous model however the confidence intervals were also larger, this may be an effect of the smaller sample size.

Table 7.4 Crude and adjusted odds ratios for Pv11 genotype and OtHV-1 status showing that UGC is over six times as likely in animals homozygous at the Pv11 locus and over 10 times as likely with animals positive for OtHV-1. Significance accepted at the 5% level.

Variable	Crude OR (95% CI)	Adjusted OR (95% CI)	P(LR-test)
***Pv11GT:HOvsHT**	5.68 (1.74,18.54)	6.51 (1.64,25.83)	0.005
****OtHV-1:Pos vs Neg**	10 (2.32,43.04)	10.12 (1.94,52.8)	0.003

*Pvll genotype, HO: homozygous; HT: heterozygous; ** OtHV-1 status as gained from the OtHV-1 specific PCR

Discussion

The results of the present study indicate that out of the areas examined, three risk factors for the presence of UGC in the CSL are apparent; Pv11 genotype, blubber thickness and OtHV-1 status. Both GLMs show a relationship with homozygostiy at the Pv11 locus and UGC, whereas the additional two microsatellite loci examined (M11a and Hg8.10) remain insignificant. Blubber thickness was not found to be significant in the smaller sample size analysed, however in the analysis involving all 113 animals it was found to be significantly associated with the presence of UGC, The discrepancy between the two models suggests that sample size affects this result. The presence of OtHV-1 as determined by the OtHV-1 specific PCR indicates a strong association with UGC.

In this study blubber thickness could be thought of as a surrogate for blubber contaminant (pollutant) level. Previous studies have demonstrated contaminant levels in blubber are associated with the presence of UGC (Ylitalo et al., 2005). The study by Ylitalo et al., (2005) measured organochlorine (OC) contaminants directly in blubber samples and identified higher levels of polychlorinated biphenyls (PCBs) in animals dying of UGC compared with those dying of other causes. In order to confirm that the use of blubber thickness is appropriate as a surrogate for the direct measurement of contaminants, an understanding of blubber dynamics is necessary. To clarify the situation, an investigation was carried out into the concentration of various persistent organic pollutants and changing blubber mass[4] in animals suffering from domoic acid toxicity. (Hall et al., 2008). The study identified that as blubber mass decreased, contaminant levels increased suggesting that they concentrate in the remaining blubber (Hall et al., 2008). Therefore although the actual levels of contaminants in the blubber of animals entered into our study were not available as the samples are still be analysed, the result that blubber thickness is significant could be due to this relationship. It could however be postulated that the higher contaminant level identified in blubber in animals suffering from UGC is a result of the thinner blubber rather than being associated with the presence of cancer. Ylitalo et al., (2005) investigated this possibility and identified that after controlling for differences in blubber thickness between control animals and

[4] Blubber mass is correlated with blubber thickness in pinnipeds as they only store fat subcutaneously (Hall et al., 2008)

those with cancer, PCB levels were still found to be significantly associated with the presence of UGC.

As large marine predators, CSLs are particularly susceptible to the effects of contaminants due to biomagnification through the food chain and numerous studies report on the levels of contaminants including OCs in tissues from CSLs (Harper et al., 2007, Stapleton et al., 2006, Kajiwara et al., 2001, Connolly and Glaser, 2002, Blasius and Goodmanlowe, 2008, Le Boeuf et al., 2002a). It is known that the waters the CSLs inhabit are historically contaminated with various industrial and agricultural chemicals including PCBs and dichloro-diphenyl-trichloroethanes (DDTs). These compounds have been identified in CSL prey species thereby allowing exposure via ingestion of contaminated food (Brown et al., 1998, Venkatesan et al., 1999, Blasius and Goodmanlowe, 2008, Jarvis et al., 2007). In female pinnipeds OC levels in the blubber are seen to fluctuate with physiological changes such as lactation and it is recognised that OCs can pass to the young both via the milk and in-utero (Debier et al., 2003b, Addison and Brodie, 1977, Bacon et al., 1992, Debier et al., 2003a, Greig et al., 2007). Lactation and pregnancy are therefore postulated to reduce contaminant load in female animals, males are clearly unable to unload contaminants in this way and higher contaminant levels in the blubber of male CSLs with UGC have been recognised in comparison to those in female animals (Ylitalo et al., 2005, Debier et al., 2003b, Nakata et al., 1995).

Contaminants can have a direct genotoxic effect predisposing the animal to cancer as mentioned in Chapter 1 (section 1.5.4). They are also seen to cause detrimental health effects in the form of immunosuppression. This effect has been reported in experiments involving harbour seals fed with fish caught in waters known to be polluted with organochlorines, where reduced activity of natural killer cells and T cell response was recognised in comparison to in control animals (de Swart et al., 1996, Ross et al., 1995). The known immunosuppressive effect of contaminants holds relevance to the present study as it could be speculated that an increased contaminant level in blubber of the CSLs suffering from UGC predisposes the animals to infection with OtHV-1.

OtHV-1 as demonstrated by the OtHV-1 specific PCR in the present study appears to be strongly associated with the presence of UGC in CSL. However this study identified

that OtHV-1 is also present in animals not suffering from the disease, therefore the involvement of other factors in cancer pathogenesis cannot be ruled out. Indeed in a study investigating the development of Kaposi sarcoma, disease was not seen to develop until co-infection with the retrovirus HIV had occurred (Jacobson et al., 2000). The only retrovirus so far identified in CSLs is a retrovirus belonging to the subfamily Spumavirinae (known as "foamy viruses") in lymph nodes in a captive CSL suffering from recurrent skin lesions (Kennedy-Stoskopf et al., 1986). Unlike the other two subfamilies in the retrovirus group (the Oncovirinae and the Lentivirinae) neoplastic disease has not been associated with infection with viruses belonging to the group of foamy viruses (Coffin, 1990, Meiering and Linial, 2001). However, other health effects have been noted as rabbits experimentally infected with Simian foamy virus type 7 showed immunosuppression for up to two weeks following inoculation, in addition to this a herpesvirus infection (of unspecified species) was diagnosed in one of the rabbits (Hooks and Detrick-Hooks, 1979). Interestingly, a herpesvirus (of unspecified species) was also isolated from the CSL infected with a foamy virus (Kennedy-Stoskopf et al., 1986).

Identifying the candidate agent responsible for initiation or promotion of cancer is challenging and becomes more difficult if that agent is no longer present. In a study investigating genital tumours in Atlantic bottlenose dolphins (*Tursiops truncates*) a γ-herpesvirus was identified via PCR carried out on DNA extracted from the tumours. The authors additionally carried out serology for papillomavirus, the results of which indicated past or present infection with a papillomavirus in 12 out of 14 animals suffering from genital tumours. In addition they reported that the remaining two animals had antibody levels near to the seropositive cutoff (Rehtanz et al., 2012). This finding led Rehtanz et al., (2012) to postulate that a "hit and run" effect may be occurring where initiation of cancer was potentially due to a papillomavirus infection that had since cleared. It was therefore concluded that it was not possible to know which viral agent (herpes or papillomavirus) potentially initiated or promoted the disease (Rehtanz et al., 2012).

Previous work investigating the involvement of papillomavirus in CSL with UGC has been undertaken in two studies (Lipscomb et al., 2000, Buckles et al., 2006). The first

study examined metastatic tissue from four animals via both southern blotting (using five different papillomavirus probes) and PCR (targeting the conserved E1 gene) but failed to find the virus (Lipscomb et al., 2000). In the second study only one out of the 15 animals with UGC investigated was found to be positive for papillomavirus via PCR (targeting the conserved L1 gene) with the papillomavirus identified as being human papillomavirus 21, suggesting contamination during tissue handling (Buckles et al., 2006). At the time of these experiments papillomavirus had not been found in pinnipeds, however in 2012 a novel papillomavirus was successfully identified in skin lesions from two CSLs (Rivera et al., 2012). In this study four nested PCRs using degenerate primers were used and targeted the conserved papillomavirus E1 gene (Rivera et al., 2012). Phylogenetic analysis identified that the novel ZcPV1 virus was closely related to canine papillomaviruses 3 and 4 (CPV3, CPV4) of genus *Chipapillomavirus*. In dogs these viruses have been associated with skin lesions and in the case of CPV3 a role in skin cancer has been suggested (Lange et al., 2009b, Lange et al., 2009a). All three studies used degenerate primers targeted to conserved papillomavirus gene regions to allow the detection of virus in the absence of exact gene sequence. Rivera et al., (2012) additionally used a nested technique which potentially increased the sensitivity of the test as a result of the increased number of amplification cycles.

UGC in CSL mirrors human cervical carcinoma in age demographic as human cervical cancer predominately occurs in adult but not necessarily aged women (Buckles et al., 2006, Gustafsson et al., 1997). Worldwide the disease in women has been strongly associated with papillomavirus 16 and 18 and this has influenced vaccine development (Munoz et al., 2004, Bosch et al., 2008, Schiffman et al., 1993). This along with the findings in the bottlenose dolphins with genital tumours indicates that the possible involvement of papillomavirus in UGC in CSL merits further investigation.

Viruses, including various herpesviruses, are reported to be present at a higher prevalence in the population than the disease they are associated with and the action of co-factors helps to explain this phenomenon (Morris et al., 1995, Monini et al., 1996, Roizman, 1995). Further to this genetic susceptibility to herpesvirus infection has been reported. A study undertaken into KSHV infection susceptibility in individuals in an

KSHV endemic area identified a genetic predisposition via a segregation analysis (Plancoulaine et al., 2003).A further study using a genome wide linkage analysis in samples from a different endemic area found a genetic locus of interest on chromosome three (Pedergnana et al., 2012). Due to the lack of information available regarding the role of the *HPSE2* gene it is not known whether it may influence susceptibility to OtHV-1 infection and the present study is too small to assist in clarifying this, as certain genotypes only appear in small numbers in the CSLs. However, the reported route of entry of herpesviruses into cells includes binding to heparan sulphate ligands, thus suggesting a potential role due to experiments identifying HPA2's high affinity to heparan sulphate (Levy-Adam et al., 2010, Shukla and Spear, 2001, Akula et al., 2001). That being said, the route of entry of KSHV, the virus identified as closely related to OtHV-1, into B cells where latency occurs is not completely understood as heparan sulphate is found to be expressed only at low levels on the surface of these cells (Jarousse et al., 2008).

CSLs homozygous at the Pv11 locus were found to be more likely to have UGC than those that were heterozygous. The location of Pv11 within the HPSE2 gene and the presence of differential labelling of HPA2 in UGC affected animals of one genotype strongly suggests that *HPSE2* plays a role in UGC in the CSL. Allele 1 was by far the most common Pv11 allele identified in this study and the only homozygous allele type that exhibited labelling of HPA2, therefore posing the question as to whether this genotype offers a protective advantage to this species. There is relatively little known about the function of the *HPSE2* gene even in humans and there is much scope for further investigation into this gene in the CSL. If future studies confirm the involvement of *HPSE2* in UGC in CSL a potential diagnostic role could be explored. The *HPSE2* gene shares sequence homology with the HPSE gene and heparanase mRNA has been investigated as a possible diagnostic marker in urine for bladder cancer in humans (Zhao et al., 2009).

Although the present study has identified *HPSE2* as potentially important in the pathogenesis of UGC in the CSL, the involvement of other genes in the vicinity cannot be discounted and this should be considered in future investigations.

Future Directions

This study is nested within a wider epidemiological study with the main focus of future directions involving Pv11 genotyping, OtHV-1 virus screening and contaminant analysis of a larger number of animals in order to increase the power of the study. The extended study would ideally contain at least 100 cases and 200 controls, all adult female animals so as to remove the confounding factors of sex and age (Hall and Gulland, 2011).

From the findings of the present study there are areas that would benefit from further scrutiny. The possible presence of genetic instability in the form of microsatellite instability and loss of heterozygosity at the Pv11 locus should be investigated in a larger number of samples and importantly following micro-dissection. More detailed analysis of the *HPSE2* gene in UGC positive and control animals by cloning and sequencing more potential isoforms along with the identification of HPA2 protein will allow greater understanding of the role of *HPSE2* in UGC. This could be complimented by quantitative PCR (qPCR) in order to identify any difference in the level of expression of a particular isoform in animals with UGC compared to those without. Investigations into the presence of OtHV-1 in CSL could be modified by implementing a qPCR as a more sensitive test in order to identify the virus. Further to this the undertaking of a second degenerate PCR, targeting the glycoprotein B gene alongside the pan-herpes PCR targeting the Dpol gene, may assist in clarifying whether a closely related virus or variant of OtHV-1 is present. Finally the potential involvement of a retrovirus either oncogenic or benign in CSL with UGC, along with serology for past or present infection with papillomavirus should be considered in future investigations.

Due to the complicated nature of neoplasia it is very unlikely that the *HPSE2* gene is the only gene associated with UGC in the CSL and carrying out genome wide studies may present other avenues of investigation into the disease. A study such as this has been undertaken previously in CSL and employed microarray technology to compare gene expression profiles in animals suffering from leptospirosis to those suffering from domoic acid toxicity (Mancia et al., 2012). The study developed a microarray for CSL by identifying probes exhibiting cross hybridisation with a canine commercial microarray and resulted in a custom array of 15000 probes. The study identified the

differential expression of 348 genes in domoic acid toxicity compared to in animals suffering from leptospirosis (Mancia et al., 2012). Implementing this technology in studying UGC in CSL may therefore be a beneficial next step in gaining a wider understanding of a genetic basis of the disease.

Conclusion

Cancer is not a "new" disease as evidence of neoplasia has been seen in both dinosaurs and in individuals from ancient civilisations (Binder et al., 2014, Rothschild et al., 2003). It is a complex multi-factorial condition where the occurrence of genetic defects ultimately results in loss of control of the cell cycle (Stanhope et al., 1964, Bunz, 2008, Hahn and Weinberg, 2002, Collins et al., 1997). The present study has aimed to answer four main questions and these will now be considered alongside a summary of the main findings:

1. Is there an association between the genotype of certain genetic markers and the presence of UGC in the CSL?

The study has identified a significant association between homozyosity at the Pv11 microsatellite and the presence of UGC in the CSL, where animals were found to be almost twice as likely to suffer from the disease if they had a homozygous Pv11 genotype (crude odds ratio: 1.62 CI: 1.04-2.58; p=0.033). No particular allele combination was identified as significant; however this was possibly the result of the sample size as a number of genotypes were only present in small numbers.

2. If an association is identified with a genetic marker and the presence of UGC does it indicate a gene of interest?

Comparative genomic studies and molecular techniques placed the Pv11 microsatellite within the *HPSE2* gene. The *HPSE2* shares 35% amino acid sequence identity with the *HPSE* gene, a gene long known to be involved in tumour metastasis. Studies so far undertaken into the role of *HPSE2* have suggested that *HPSE2* does play a role in cancer however the exact function is unknown. Although the present study failed to identify differences between *HPSE2* expression products in animals of different Pv11 genotypes and disease state, differential labelling of the HPA2 protein in urogenital tract tissue was evident. Labelling of HPA2 was only present in CSL suffering from UGC of only one Pv11 homozygous genotype (1,1). The presence of differential labelling strongly suggests that Pv11 and the *HPSE2* gene are linked and therefore indicates that *HPSE2* is a gene of interest in the development of UGC in this species.

3. Does genetic instability occur in CSL with UGC?

Microsatellite instability (MI) in the form of contraction of the repeat CA unit was identified in three samples; two control animals and one UGC positive animal. The limited instability identified suggests that MI at the Pv11 locus is not a common change. Additionally there is the potential that the MI identified is a result of *Taq* polymerase slippage during PCR. Loss of heterozygosity (LOH) at the Pv11 locus was identified in one UGC positive animal (out of the seven UGC positive heterozygotes and 26 control heterozygotes examined). Clarification of the situation as to whether genetic instability is a feature of the Pv11 locus is still required and repetition of the experiments investigating the presence of MI and LOH at the Pv11 locus should be carried out with a larger sample size alongside implementing micro-dissection to collect samples.

4. Is the presence of herpesvirus associated with the occurrence of UGC?

The two methods employed to identify the presence of herpesvirus yielded very different results. Although sequences gained from the pan herpes PCR identified a single virus (OtHV-1) it also identified a higher prevalence in the control animals than the OtHV-1 specific PCR. This resulted in the lack of a significant association between the presence of OtHV-1 and UGC being identified with the pan herpes screen ($p=0.788$), this was opposed to a strongly significant association identified with the OtHV-1 specific PCR ($p=0.0002$). The contradictory results could be explained by potential differences in sensitivity of the tests or presence of a variant of the OtHV-1 virus being detected by the pan herpes screen. Quantitative molecular techniques along with more detailed sequencing of the OtHV-1 virus is therefore required to assist in further understanding the relationship between herpesvirus and UGC in the CSL.

Alongside the identification of a genetic basis and potential infectious aetiology to UGC in the CSL, further analysis identified thinner blubber thickness to be a risk factor (with blubber thickness being considered a surrogate for contaminant level). Therefore the multi-factorial nature of cancer is clearly recognised in UGC in the CSL with more than one risk factor being identified. Thus strongly supporting the initial general hypothesis:

"Urogenital carcinoma in the California sea lion has a multi-factorial aetiology including a genetic and infectious basis"

The importance of understanding UGC in CSL has heightened since the discovery of the disease in a South American fur seal. The fact that it has been recognised in an otariid species other than the CSL suggests that other otariids including those considered as an endangered or threatened species may also be at risk.

Investigating diseases in animals, especially wildlife, holds many difficulties not encountered in disease investigation in humans, however the parallels that exist to human diseases show it to be an area worthy of further exploration (Airley, 2012, Paoloni and Khanna, 2008). The present study has identified what may be the first cancer gene in a wildlife species. These findings offer support for further studies into *HPSE2*s involvement in cancers in other species including in humans, where investigations involving this gene are still in their infancy.

In light of recent world events such as the Deepwater Horizon oil spill in the Gulf of Mexico, resulting in a large environmental exposure to carcinogens, studies into wildlife cancer may become more important. Effects on wildlife have already been noted in the form of endocrine disruption in bottlenose dolphins in the area (Schwacke et al., 2014). The role that marine mammals are believed to play as sentinels for the health of the marine environment reinforces the importance of understanding what factors challenge their health as it may well highlight risks to our own.

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Appendix A

Genotypes of the 113 animals in the study at three microsatellite loci including cause of death (DAT: domoic acid toxicity; Misc. Inf.: Miscellaneous infection and inflammation; Undet.: Undetermined; Other Neo. Neoplasia other than urogenital carcinoma; UGC: urogenital carcinoma). Genotype derived from skin DNA.

Animal ID	Pv11 genotype	M11a genotype	Hg8.10 genotype	Cause of death
9086 (1)	1,3	2,8	6,7	DAT
9113 (7)	2,4	3,4	2,7	DAT
9114 (2)	1,1	8,8	2,5	DAT
9120 (20)	1,2	3,4	2,4	DAT
9184 (14)	1,1	7,8	5,6	DAT
9190	2,3	1,1	4,4	DAT
9193 (27)	2,4	1,8	2,5	DAT
9196 (78)	1,1	7,8	4,6	DAT
9198 (21)	1,4	1,8	4,6	DAT
9201 (6)	1,4	7,7	5,5	DAT
9205 (19)	2,4	1,8	2,5	DAT
9208 (11)	1,4	3,8	2,4	DAT
9212 (10)	1,2	8,8	4,7	DAT
9221 (16)	1,3	7,8	2,4	DAT
9245 (46)	1,2	7,7	5,6	Misc. Inf.
9254 (3)	2,5	1,8	2,5	Misc. Inf.
9260 (13)	1,2	3,7	4,4	DAT
9299 (24)	1,2	2,7	4,5	DAT
9303 (36)	1,1	7,7	4,4	DAT
9304 (47)	1,2	1,3	5,5	DAT
9315 (48)	1,3	6,8	5,5	DAT
9320 (29)	1,4	4,9	5,6	DAT
9356 (32)	3,4	1,2	4,5	DAT
9463 (28)	1,1	4,4	5,5	Misc. Inf.
9468 (5)	1,4	2,7	4,4	Misc. Inf.
9574 (40)	1,2	2,8	4,5	DAT
9597 (43)	2,2	7,9	2,6	DAT
9694 (9)	1,2	3,7	2,4	Misc. Inf.
9755 (35)	1,2	8,8	7,7	Undet.

Animal	Pv11 genotype	M11a genotype	Hg8.10 genotype	Cause of death
9764	1,3	1,8	5,5	DAT
9779 (4)	1,4	1,3	2,5	Misc. Inf.
9801	1,4	3,7	4,5	DAT
9821 (72)	1,2	8,8	4,4	DAT
9866 (44)	1,4	4,7	2,6	DAT
9871 (23)	3,5	3,8	2,4	DAT
9881	1,2	7,7	2,3	DAT
9906 (26)	1,2	1,8	5,7	DAT
9907 (37)	1,2	3,3	5,6	DAT
7819 (75)	2,2	3,8	4,4	DAT
9325 (76)	1,1	8,8	2,5	DAT
7290	2,4	7,8	4,5	DAT
7295	1,2	3,7	4,4	Other Neo.
7371	2,4	4,7	5,5	Other Neo.
7147	1,4	2,8	7,7	DAT
7329	1,2	3,3	2,2	Misc. Inf.
7159	4,4	7,8	4,5	DAT
7131	3,3	8,8	4,5	DAT
7741	3,4	3,8	5,7	DAT
7750	2,2	7,7	4,5	Undet.
8029 (84)	2,2	3,7	4,7	DAT
6863	1,2	3,8	2,4	Lept.
8041	1,3	3,8	5,5	DAT
8645	1,2	2,4	2,4	Trauma
9100 (77)	1,1	2,4	2,4	DAT
9079	1,4	2,7	2,5	DAT
8901	1,2	3,8	4,6	Misc. Inf.
8999	1,4	1,8	5,7	Trauma
8795	1,2	8,8	5,7	DAT
8958 (79)	1,1	7,8	4,6	DAT
9112	1,2	3,4	2,7	DAT
9157	1,2	3,8	4,4	DAT
9164	1,3	1,7	5,5	DAT
8722	1,4	7,8	5,5	DAT
9155	1,2	4,4	5,7	DAT

Animal	Pv11 genotype	M11a genotype	Hg8.10 genotype	Cause of death
9255	1,2	1,2	2,4	Misc. Inf.
9008 (80)	1,1	1,7	4,6	DAT
9032	1,2	8,8	5,7	Misc. Inf.
9534	1,4	3,8	6,6	Other Neo.
7977 (17)	1,2	1,8	2,6	Other Neo.
7594	2,3	1,8	5,7	DAT
7919	1,2	3,3	2,5	UGC
7997 (68)	1,1	7,8	5,5	UGC
8059 (30)	3,3	8,8	4,5	UGC
8431 (69)	1,1	1,8	4,5	UGC
8489 (15)	1,2	8,8	5,5	UGC
8921 (33)	4,4	4,8	2,4	UGC
8992 (22)	1,2	3,8	4,5	UGC
9107 (12)	1,3	4,7	4,7	UGC
9225 (18)	1,4	1,4	5,7	UGC
9251	1,4	4,8	7,7	UGC
9333 (38)	1,3	8,8	1,4	UGC
9339 (70)	3,3	2,4	5,5	UGC
9572 (71)	3,3	8,8	5,5	UGC
9724 (25)	2,2	3,4	2,5	UGC
9757 (39)	1,1	1,1	4,5	UGC
9770 (42)	2,2	1,8	5,6	UGC
9804 (81)	1,1	2,4	4,5	UGC
9827 (41)	1,1	1,8	5,5	UGC
9853 (31)	1,2	7,8	6,7	UGC
9904 (45)	1,3	4,7	4,5	UGC
9911 (34)	1,1	8,8	4,7	UGC
7867 (73)	3,3	8,8	4,6	UGC
7972 (74)	1,1	7,7	2,4	UGC
7278	1,2	6,8	4,5	UGC
7495	1,3	4,8	4,5	UGC
7468 (83)	1,1	2,7	4,7	UGC
7506	1,2	3,3	4,5	UGC
7379	1,4	2,8	4,6	UGC
7380	1,4	2,7	4,5	UGC

Animal	Pv11 genotype	M11a genotype	Hg8.10 genotype	Cause of death
7425	1,2	1,8	6,7	UGC
7325	1,2	4,6	2,7	UGC
7140 (82)	1,1	1,8	2,5	UGC
6370	1,1	7,8	4,4	UGC
7150	2,4	1,7	2,5	UGC
7720	1,4	1,8	4,5	UGC
7892	1,3	3,8	5,6	UGC
7766	2,4	8,8	4,4	UGC
8018	1,2	3,8	2,5	UGC
8039	1,3	7,8	6,6	UGC
8068	2,4	3,8	2,6	UGC
7755	1,2	3,4	4,4	UGC
8673	1,4	5,8	4,4	UGC
9091	1,2	2,7	2,4	UGC

Appendix B

Details of the Pv11 genotype of the 270 additional samples consisting of 66 animals with urogenital carcinoma (UGC) and 204 without UGC (Control) genotyped by Dr Karina Acevedo-Whitehouse. (Two additional alleles were identified by Dr Acevedo-Whitehouse and are detailed in the table as alleles 'A' and 'B'). Genotyped derived from skin DNA.

Animal	Pv11 genotype	Urogenital carcinoma status
Z1132	A, A	Control
Z104	B,1	Control
Z1234	1,1	Control
Z797	1,1	Control
Z251	1,1	Control
Z805	1,1	Control
Z792	1,1	Control
Z864	1,1	Control
Z1408	1,1	Control
Z148	1,1	Control
Z1133	1,1	Control
Z798	1,1	Control
Z1031	1,1	Control
Z353	1,1	Control
Z1211	1,1	Control
Z800	1,1	Control
Z799	1,1	Control
Z1396	1,1	Control
Z809	1,1	Control
Z499	1,1	Control
Z1232	1,1	Control
Z471	1,1	Control
Z1293	1,1	Control
Z263	1,1	Control
Z1220	1,1	Control
Z1238	1,1	Control
Z833	1,1	Control
Z1212	1,1	Control
Z041	1,1	Control

Animal	Pv11 genotype	Urogenital carcinoma status
Z1100	1,1	Control
Z1060	1,1	Control
Z811	1,1	Control
Z870	1,1	Control
Z1079	1,1	Control
Z485	1,1	Control
Z1030	1,1	Control
Z015	1,1	Control
Z1111	1,1	Control
Z1153	1,1	Control
Z008	1,1	Control
Z075	1,1	Control
Z270	1,1	Control
Z112	1,1	Control
Z1379	1,1	Control
Z476	1,1	Control
Z961	1,1	Control
Z1335	1,1	Control
Z143	1,1	Control
Z277	1,1	Control
Z967	1,1	Control
Z124	1,1	Control
Z252	1,1	Control
Z158	1,1	Control
Z484	1,1	Control
Z1308	1,2	Control
Z1160	1,2	Control
Z1248	1,2	Control
Z802	1,2	Control
Z1383	1,2	Control
Z871	1,2	Control
Z944	1,2	Control
Z260	1,2	Control
Z1214	1,2	Control
Z268	1,2	Control

Animal	Pv11 genotype	Urogenital carcinoma status
Z818	1,2	Control
Z822	1,2	Control
Z407	1,2	Control
Z249	1,2	Control
Z1147	1,2	Control
Z1129	1,2	Control
Z435	1,2	Control
Z245	1,2	Control
Z812	1,2	Control
Z789	1,2	Control
Z004	1,2	Control
Z1219	1,2	Control
Z1110	1,2	Control
Z1205	1,2	Control
Z951	1,2	Control
Z945	1,2	Control
Z1155	1,2	Control
Z1235	1,2	Control
Z863	1,2	Control
Z796	1,2	Control
Z1134	1,2	Control
Z1237	1,2	Control
Z803	1,2	Control
Z162	1,2	Control
Z121	1,2	Control
Z1154	1,2	Control
Z092	1,2	Control
Z831	1,2	Control
Z1298	1,2	Control
Z1083	1,2	Control
Z111	1,2	Control
Z007	1,2	Control
Z145	1,2	Control
Z218	1,2	Control
Z423	1,2	Control

Animal	Pv11 genotype	Urogenital carcinoma status
Z841	1,2	Control
Z1123	1,2	Control
Z113	1,2	Control
Z1381	1,2	Control
Z1143	1,2	Control
Z1120	1,2	Control
Z187	1,2	Control
Z189	1,2	Control
Z403	1,2	Control
Z207	1,2	Control
Z1376	1,2	Control
Z832	1,2	Control
Z1386	1,3	Control
Z868	1,3	Control
Z1240	1,3	Control
Z038	1,3	Control
Z891	1,3	Control
Z037	1,3	Control
Z790	1,3	Control
Z022	1,3	Control
Z1159	1,3	Control
Z1034	1,3	Control
Z1131	1,3	Control
Z1310	1,3	Control
Z823	1,3	Control
Z185	1,3	Control
Z062	1,3	Control
Z469	1,3	Control
Z147	1,3	Control
Z177	1,3	Control
Z119	1,3	Control
Z462	1,4	Control
Z942	1,4	Control
Z125	1,4	Control
Z026	1,4	Control

Animal	Pv11 genotype	Urogenital carcinoma status
Z810	1,4	Control
Z807	1,4	Control
Z1324	1,4	Control
Z470	1,4	Control
Z257	1,4	Control
Z1204	1,4	Control
Z793	1,4	Control
Z947	1,4	Control
Z057	1,4	Control
Z359	1,4	Control
Z1239	1,4	Control
Z1397	1,4	Control
Z816	1,4	Control
Z016	1,4	Control
Z791	1,4	Control
Z990	1,4	Control
Z1331	1,4	Control
Z097	1,4	Control
Z069	1,4	Control
Z133	1,4	Control
Z1325	1,4	Control
Z795	1,5	Control
Z475	1,5	Control
Z455	1,5	Control
Z1229	2,2	Control
Z1236	2,2	Control
Z395	2,2	Control
Z153	2,2	Control
Z939	2,2	Control
Z804	2,2	Control
Z1231	2,2	Control
Z472	2,2	Control
Z247	2,2	Control
Z817	2,2	Control
Z946	2,2	Control

Animal	Pv11 genotype	Urogenital carcinoma status
Z1319	2,2	Control
Z001	2,2	Control
Z1177	2,2	Control
Z1051	2,2	Control
Z049	2,2	Control
Z1314	2,2	Control
Z031	2,3	Control
Z806	2,3	Control
Z045	2,3	Control
Z867	2,3	Control
Z262	2,3	Control
Z869	2,3	Control
Z826	2,3	Control
Z330	2,3	Control
Z463	2,3	Control
Z1375	2,3	Control
Z450	2,3	Control
Z814	2,4	Control
Z278	2,4	Control
Z409	2,4	Control
Z042	2,4	Control
Z866	2,4	Control
Z1198	2,4	Control
Z454	2,4	Control
Z1103	2,4	Control
Z838	2,4	Control
Z018	3,3	Control
Z815	3,3	Control
Z442	3,3	Control
Z1294	3,4	Control
Z003	3,4	Control
Z100	3,4	Control
Z1242	3,5	Control
Z024	4,4	Control
Z801	4,4	Control

Animal	Pv11 genotype	Urogenital carcinoma status
Z139	B,1	UGC
Z077	1,1	UGC
Z1116	1,1	UGC
Z265	1,1	UGC
Z827	1,1	UGC
Z1048	1,1	UGC
Z829	1,1	UGC
Z954	1,1	UGC
Z808	1,1	UGC
Z006	1,1	UGC
Z1380	1,1	UGC
Z105	1,1	UGC
Z835	1,1	UGC
Z181	1,1	UGC
Z443	1,1	UGC
Z273	1,1	UGC
Z141	1,1	UGC
CSL33	1,1	UGC
CSL223	1,1	UGC
CSL292	1,1	UGC
Z1312	1,2	UGC
Z426	1,2	UGC
Z445	1,2	UGC
Z012	1,2	UGC
Z1311	1,2	UGC
Z1384	1,2	UGC
Z172	1,2	UGC
CSL221	1,2	UGC
Z096	1,3	UGC
Z482	1,4	UGC
Z819	1,4	UGC
Z788	1,4	UGC
Z1151	1,4	UGC
Z830	1,4	UGC
Z825	1,4	UGC

Animal	Pv11 genotype	Urogenital carcinoma status
Z828	1,4	UGC
Z013	1,4	UGC
Z1385	1,4	UGC
Z464	2,2	UGC
Z821	2,2	UGC
Z1382	2,2	UGC
Z127	2,2	UGC
Z101	2,2	UGC
CSL183	2,2	UGC
CSL310	2,2	UGC
Z845	2,3	UGC
Z824	2,3	UGC
Z839	2,3	UGC
Z1387	2,4	UGC
Z079	2,4	UGC
Z837	2,4	UGC
Z091	2,4	UGC
Z836	2,4	UGC
Z425	2,4	UGC
Z083	2,4	UGC
CSL198	2,4	UGC
Z775	2,4	UGC
Z005	3,3	UGC
CSL241	3,3	UGC
CSL284	3,3	UGC
Z844	3,3	UGC
CSL177	3,4	UGC
CSL371	3,4	UGC
CSL16	4,4	UGC
Z117	4,4	UGC
CSL243	4,4	UGC

Appendix C

Results of the pan-herpes PCR and OtHV-1 specific PCR. An undetermined result indicates an inconclusive PCR. All pan-herpes PCR positive samples were identified as OtHV-1 on sequencing. Cause of death: - DAT: domoic acid toxicity; Misc. Inf.: Miscellaneous infection and inflammation; Undet.: Undetermined; Other Neo. Neoplasia other than urogenital carcinoma; UGC: urogenital carcinoma). (*Genotype determined from DNA from lower genital tract tissue).

Animal ID	Pv11 genotype	Tissue	Pan-herpes PCR	OtHV-1 specific PCR	Cause of death
9086 (1)	1,3	Cervix	Positive	Negative	DAT
9114 (2)	1,1	Cervix	Positive	Negative	DAT
9254 (3)	2,5	Cervix	Positive	Negative	Misc. Inf.
9779 (4)	1,4	Cervix	Positive	Negative	Misc. Inf.
9468 (5)	1,4	Cervix	Positive	Negative	Misc. Inf.
9201 (6)	1,4	Cervix	Positive	Negative	DAT
9113 (7)	2,4	Cervix	Negative	Negative	DAT
9274 (8)	2,4*	Cervix	Undetermined	Negative	DAT
9694 (9)	1,2	Cervix	Negative	Negative	Misc. Inf.
9212 (10)	1,2	Cervix	Undetermined	Negative	DAT
9208 (11)	1,4	Cervix	Positive	Negative	DAT
9260 (13)	1,2	Cervix	Positive	Negative	DAT
9184 (14)	1,1	Cervix	Positive	Negative	DAT
9221 (16)	1,3	Cervix	Negative	Negative	DAT
7977 (17)	1,2	Cervix/vagina	Negative	Negative	Other Neo.
9205 (19)	2,4	Cervix	Negative	Negative	DAT
9120 (20)	1,2	Cervix	Negative	Negative	DAT
9198 (21)	1,4	Cervix	Positive	Negative	DAT
9871 (23)	3,5	Cervix	Positive	Positive	DAT
9299 (24)	1,2	Cervix	Positive	Negative	DAT
9906 (26)	1,2	Cervix	Positive	In	DAT
9193 (27)	2,4	Cervix	Undetermined	Negative	DAT
9463 (28)	1,1	Cervix	Undetermined	Negative	Misc. Inf.
9320 (29)	1,4	Cervix	Positive	Negative	DAT
9356 (32)	3,4	Cervix	Negative	Positive	DAT
9755 (35)	1,2	Cervix	Positive	Negative	Undet.
9303 (36)	1,1	Cervix	Undetermined	Negative	DAT

Animal ID	Pv11 genotype	Tissue	Pan-herpes PCR	OtHV-1 specific PCR	Cause of death
9907 (37)	1,2	Cervix	Negative	Negative	DAT
9574 (40)	1,2	Cervix	Negative	Negative	DAT
9597 (43)	2,2	Cervix	Positive	Negative	DAT
9866 (44)	1,4	Cervix	Positive	Negative	DAT
9245 (46)	1,2	Cervix	Positive	Negative	Misc. Inf.
9304 (47)	1,2	Cervix	Negative	Negative	DAT
9315 (48)	1,3	Cervix	Negative	Negative	DAT
9821 (72)	1,2	Cervix	Positive	Negative	DAT
7819 (75)	2,2	Cervix/vagina	Positive	Negative	DAT
9325 (76)	1,1	Cervix/vagina	Positive	Negative	DAT
9100 (77)	1,1	Cervix	Negative	Negative	DAT
9196 (78)	1,1	Cervix	Negative	Negative	DAT
8958 (79)	1,1	Cervix	Negative	Negative	DAT
9008 (80)	1,1	Cervix	Negative	Positive	DAT
8029 (84)	2,2	Cervix	Negative	Negative	DAT
9107 (12)	1,3	Cervix	Negative	Positive	UGC
8489 (15)	1,2	Cervix	Negative	Positive	UGC
9225 (18)	1,4	Cervix	Negative	Negative	UGC
8992 (22)	1,2	Cervix	Positive	Negative	UGC
9724 (25)	2,2	Cervix	Negative	Negative	UGC
8059 (30)	3,3	Cervix/vagina	Negative	Negative	UGC
9853 (31)	1,2	Cervix	Negative	Positive	UGC
8921 (33)	4,4	Cervix/vagina	Positive	Undetermined	UGC
9911 (34)	1,1	Prox. Vagina	Positive	Positive	UGC
9333 (38)	1,3	Cervix	Undetermined	Positive	UGC
9757 (39)	1,1	Cervix	Negative	Positive	UGC
9827 (41)	1,1	Cervix	Positive	Positive	UGC
9770 (42)	2,2	Cervix	Positive	Positive	UGC
9904 (45)	1,3	Cervix	Positive	Negative	UGC
7997 (68)	1,1	Cervix	Negative	Negative	UGC
8431 (69)	1,1	Cervix/vagina	Negative	Negative	UGC
9339 (70)	3,3	Cervix	Negative	Negative	UGC
9572 (71)	3,3	Cervix/vagina	Positive	Positive	UGC
7867 (73)	3,3	Cervix/vagina	Positive	Negative	UGC

Animal ID	Pv11 genotype	Tissue	Pan-herpes PCR	OtHV-1 specific PCR	Cause of death
7972 (74)	1,1	Cervix	Negative	Negative	UGC
9804 (81)	1,1	Cervix	Positive	Negative	UGC
7140 (82)	1,1	Prox. Vagina	Positive	Positive	UGC
7468 (83)	1,1	Cervix/vagina	Positive	Positive	UGC

Appendix D

Standard experimental methods:

Chapter 3: - Southern blot

Performing the Southern blot using the DIG method requires a number of steps: 1. DNA extraction, 2. Restriction digestion and DNA separation, 3. Gel preparation and blotting, 4. Preparation of probes, 5. Hybridization and 6. Detection, these are detailed below:

(1) DNA extraction

Genomic DNA previously extracted from CSL and harbour seal tissue at The Pirbright Institute (Compton, UK), were used in this study. All tissues were stored at -80°C and following extraction DNA was stored at -20°C.

(2) Restriction digest and DNA separation

The restriction digest was carried out by combining 40 µl of genomic DNA, 10 µl of reaction buffer (NEBuffer3.1 and NEBuffer2.1 for BamHI and HindIII respectively both obtain from New England Biolabs Hitchin, UK), 8 µl of either BamHI or HindIII and 42 µl of water followed by incubating the mixture overnight at 37°C. The digest reactions were loaded onto a 1% TBE agarose gel with 2 µl loading dye, BlueJuice™ Gel Loading Buffer (Invitrogen, Paisley, UK) along with 5 µl of DIG-labelled DNA molecular weight marker (Roche Applied Science, Mannheim, Germany). Gel electrophoresis was carried out at 100 V until the blue dye was seen to reach the bottom of the gel. The corner of the gel was nicked to prevent incorrect orientation and the gel was post stained by briefly submerging it in 1 litre of distilled water containing 25 µl of 10 mg/ml ethidium bromide (Sigma-Aldrich Ltd, Gillingham, UK). The gel was then photographed under UV light with a ruler alongside to allow future size determination.

(3) Gel preparation and blotting

Prior to transferring the DNA onto a membrane (blotting) it was necessary to depurinate and denature the DNA to assist in transfer of DNA to the membrane and to break the DNA into single strands allowing future hybridisation of probes (Brown, 2001).

Depurination was undertaken by placing the gel in approximately 250 ml of 0.25 M HCl for 10-15 min, the liquid was agitated gently by rocking, and the gel was then rinsed in distilled water for 5 min before the denaturation step. The DNA was denatured by placing the gel in approximately 250 ml of a buffer of 1.5 M NaCl and 0.5 M NaOH for 15-20 min, this step was repeated following replacement with fresh buffer. The gel was rinsed again for 5 min in distilled water before being rocked for 30 min in a solution of 0.5 M Tris 7.5 and 1.5 M NaCl, the solution was renewed and the gel rocked again for 30 min. The gel was then rinsed for 10 min in 20x Saline-Sodium Citrate (SSC) buffer (Sigma-Aldrich, Gillingham, UK).

To enable blotting a piece of Whatman 3MM paper (GE Healthcare, Little Chalfont, UK) was placed on a platform in a tray containing 20x SSC buffer (Sigma-Aldrich, Gillingham, UK), the Whatman paper was then left to draw up the buffer until it was soaked. The gel was positioned on top of the soaked Whatman paper and a piece of Hybond-N+ (GE Healthcare, Little Chalfont, UK) positively charged nylon membrane, cut to the size of the gel, was placed on the gel. Parafilm was placed around the gel and two more pieces of soaked Whatman paper placed on the membrane followed by two dry pieces and then a stack of paper towels. Finally a tray with a weight on it was put on top (Figure 3.1). The blot was left overnight to allow the transfer of DNA onto the membrane by capillary action.

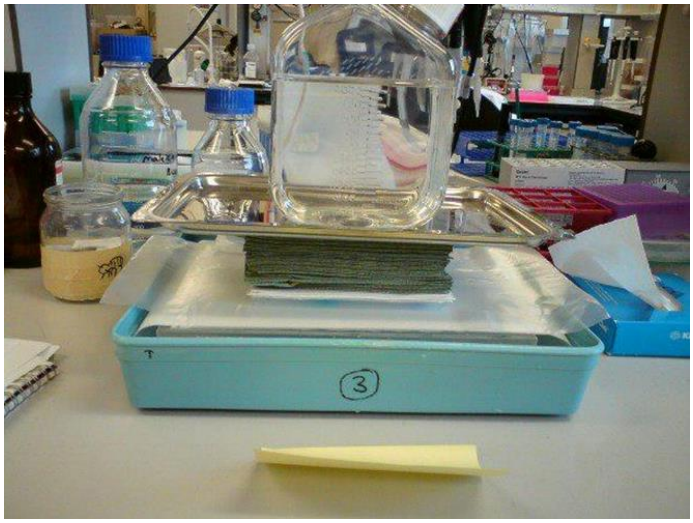

Fig. D.1 Southern blot. Tray containing SSC buffer with Hybond-N+ membrane on the agarose gel, allowing transfer of DNA via capillary action.

Following overnight blotting the DNA was fixed onto the membrane by UV crosslinking in a Stratalinker (Stratgene UV Stratlinker 1800, La Jolla, USA) set at

120,000 microjoules/cm^2. The membrane was then rinsed in distilled water and left to air dry. Once dry it was placed between two sheets of dry Whatman paper in a sealed bag and stored until it was required at 4°C.

(4) Preparation of probes

In order to label the probes, 300 ng of template DNA probe was added to an Eppendorf containing a total reaction volume of 16 µl (made up with double distilled water) and denatured by placing it in a boiling water bath for 10 min followed by chilling on ice. A vial of DIG-High Prime (Roche Applied Science, Mannheim, Germany) was mixed and 4 µl of which was added to the DNA and mixed and briefly centrifuged. The mixture was incubated overnight at 37 °C before the reaction was stopped by heating it to 65°C for 10 min. The quantity of labelled probe is estimated by the manufacturer to give an expected average yield of 2000 ng.

(5) Hybridization

Hybridization was carried out separately for both probes using the same protocol. Prior to applying the probe a pre-hybridization step was carried out to reduce background staining where the membrane was incubated for 1 hr at 37°C in a sealed bag containing 30 ml of DIG Easy Hyb solution (Roche Applied Science, Mannheim, Germany). To make a hybridization solution approximately 500 ng of labelled probe was added to 50 µl of water in an Eppendorf and placed in boiling water for 10 min followed by chilling on ice. A further 30 ml of DIG Easy Hyb solution (Roche Applied Science, Mannheim, Germany) was warmed to 37 °C and the labelled probe was added to this and the solution mixed by inverting. The pre-hybridization solution was then removed from the bag containing the membrane and replaced with the hybridization solution, the bag was re-sealed removing any air bubbles. The bag was placed in a water bath at 42 °C (with gentle rocking) overnight.

Following overnight incubation the hybridization solution was removed from the membrane and the membrane placed in a tray with 200 ml of Low Stringency Buffer; 2x SSC (Sigma-Aldrich, Gillingham, UK) with 0.1% sodium dodecyl sulfate (SDS), (Sigma-Aldrich, Gillingham, UK), for 5 min with shaking at room temperature, this was repeated with fresh buffer. High Stringency Buffer (0.5x SSC (Sigma-Aldrich,

Gillingham, UK) with 0.1% SDS, Sigma-Aldrich, Gillingham, UK) was pre-warmed to 68 °C and following the low stringency washes the membrane was placed in the heated high stringency buffer for 15 min, this was repeated with fresh buffer. The stringency washes were undertaken to prevent non-specific binding of the probe. Finally the membrane was rinsed for 1-5 min in 100 ml of Washing Buffer (0.1 M Maleic acid, 0.15 M NaCl; pH 7.5; 0.3% (v/v) Tween 20).

(6) Detection

Prior to chemiluminescent detection the membrane was incubated for 30 min in 100 ml Blocking Solution (10 x Blocking stock solution, Roche Applied Science, Mannheim, Germany, diluted 1:10 with [5]maleic acid buffer) with shaking in order to reduce background. This was followed by 30 min in 20 ml Antibody Solution (Anti-Digoxigenin-AP, Roche Applied Science, Mannheim, Germany, diluted 1:10,000 with Blocking Solution prepared as above) with shaking to apply the anti-DIG-alkaline phosphatase before being washed twice for 15 min in 100 ml of Washing Buffer (0.1 M Maleic acid, 0.15 M NaCl; pH 7.5; 0.3% (v/v) Tween 20). The membrane was then placed in 20 ml of Detection Buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5) for 2-5 min. After which the membrane was placed in a hybridization bag and 1 ml CSPD ready-to-use (Roche Applied Science, Mannheim, Germany) was applied. The bag was closed but not sealed in order to remove air bubbles and spread out the CSPD. The membrane was incubated at room temperature for 5 min. The excess liquid was then removed from the bag; the bag was sealed and incubated at 37 °C for 10 min. Detection of the DIG labelled probes was achieved via exposure to an x-ray film for 15 min. The film was then examined to identify the location of the hybridised probes

Following detection of hybridisation of the first probe, the membrane was "stripped" in order to remove the first probe before application of the second. Stripping was carried out by rinsing the membrane in double distilled water for 1 min followed by washing the membrane twice for 15 min at 37°C in Stripping Buffer; 0.2 M NaOH containing 0.1% SDS (Sigma-Aldrich, Gillingham, UK) and completed by rinsing the membrane twice with 2 x SSC (Sigma-Aldrich, Gillingham, UK).

[5] Malic acid buffer: 0.1 M Maleic acid, 0.15 M NaCl; adjusted to pH 7.5 with NaOH pellets

Chapter 5: - Identification of isoforms by cloning

In order to identify isoforms present cloning of PCR products followed by sequencing was undertaken using the following steps; (1) Preparation of insert, (2) Ligation, (3) Transformation, and Identification of positive transformants and (4) Sequencing and isoform analysis. The methods used as detailed below.

(1) Preparation of Insert

The three largest bands were chosen for gel extraction as they were consistently the strongest bands in all the samples, closest to the expected product size and therefore likely to be full length or the largest splice variant of *HPSE2*. Gels were visualised in a UV light box and a clean scalpel was used to extract the chosen bands. All of the gel containing a chosen band was placed into a 15 ml falcon tube and gel extraction carried out using a QIAquick gel extraction kit (Qiagen, Crawley, UK). Due to the higher quantity of gel, the protocol was modified by adding QG Buffer until the gel was covered instead of the measured amount. This resulted in a high quantity of liquid which was repeatedly put through the spin filter until it was all gone; the extraction was then continued as per manufacturer's instructions. The DNA was eluted with 30 µl of RNase free water (Qiagen, Crawley, UK) and the concentration of eluted DNA was measured on a Nanodrop spectrophometer (ND-1000, Thermo Fisher Scientific, Wilmington, USA).

(2) Ligation

The plasmid pGEM-T easy vector was used to amplify the insert. Plasmids are extra-chromosomal circular pieces of DNA that are replicated during cell replication therefore when used as cloning vectors the genetic material of interest is replicated also (Lodish et al., 2000). To prepare the ligation reaction the pGEM-T easy vector (Promega, Southampton, UK) was centrifuged and the ligation buffer mixed by vortex. Ligation buffer (5 µl) (Promega, Southampton, UK) along with 1 µl of vector, approximately 100 ng of insert, 1 µl of T4 DNA ligase and RNase free water (Qiagen, Crawley, UK) to 10 µl were combined in 0.2 ml PCR tubes. Although it was preferred to have 100 ng of insert for the ligation reaction this was not possible in all cases as the DNA concentration from the gel extraction was too low, in these cases the maximum volume

of 3 µl of gel extraction product was added instead. The reactions were mixed by pipetting gently and incubated at 4°C overnight.

(3) Transformation and Identification of Positive Transformants

Transformation describes the uptake of extra DNA such as plasmids into bacterial cells (Lorenz and Wackernagel, 1994). When used as cloning vectors plasmids can be transferred to cells by either electro-transformation or chemical transformation and in either case the target cells must be suitably competent as reviewed by Trevors, 1998 (Trevors, 1998). In order to carry out the transformation reaction the ligation reaction was centrifuged briefly and a vial of TOP10 cells (One Shot® TOP10 Chemically Competent E. coli cells, Invitrogen, Paisley, UK) thawed on ice. Once the cells had thawed all of the ligation reaction was added to them. The mixture was flicked gently to mix and placed on ice for 20 min. Once the incubation time on ice had been completed the cells were heat shocked in a water bath heated to exactly 42°C for 45-50 s. The tubes were immediately returned to ice for 2 min, followed by the addition of 950 µl of SOC medium (Invitrogen, Paisley, UK) at room temperature. The tubes were then incubated for 1.5 h with shaking (150 rpm) at 37°C. While the tubes were incubated two Luria-Bertani agar (LB agar, Sigma-Aldrich Ltd, Gillingham, UK) plates containing 100 µg/ml ampicillin (Sigma-Aldrich Ltd, Gillingham, UK) and 80 µg/ml 5-Bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) (VWR International Ltd, Lutterworth, UK) were dried. After the incubation step 200 µl of the reaction was plated onto one of the X-gal/amp plates and the remainder plated onto the second plate.

The pGEM-T Easy vector has two important features; it carries a selection marker along with a reporter gene. The selection marker is in the form of an ampicillin resistance gene (β-lactamase) which results in only those colonies carrying the plasmid being able to survive when challenged with ampicillin in the agar (Wong, 2006, Preston, 2003). The second important feature of this plasmid is the presence of a functioning *lacZ* gene. The *lacZ* gene acts as a reporter gene allowing the identification of potential positive transformants via colour screening of bacterial colonies. The *lacZ* gene codes for the enzyme β-galactosidase, which acts upon the β-galactoside X-gal incorporated into the agar resulting in a blue colour. Ligation of the insert into the vector disrupts the coding

ability of the *LacZ* gene, therefore preventing the production of β-galactosidase and the subsequent production of white colonies (Burn, 2012).

Although the presence of white colonies suggest cloning success it is still necessary to confirm that the insert cloned is the one of interest. In order to do this plasmid preparations followed by diagnostic digests were carried out. The plasmid vector has a number of restriction enzyme sites which are located either side of the site of insertion. By carrying out a restriction digest and releasing the insert it can be established if the DNA fragment cloned is of appropriate size.

Sixteen white colonies were isolated per sample from the two X-gal/amp plates using a sterile culture loop and sub-cultured into sterile universal tubes containing 5 ml Luria-Bertani broth (LB broth Sigma-Aldrich Ltd, Gillingham, UK) supplemented with 100 µg/ml ampicillin (Sigma-Aldrich Ltd, Gillingham, UK). The tubes were incubated overnight at 37°C with shaking at 150 rpm. A PureLink Quick Plasmid Miniprep Kit (Invitrogen, Paisley, UK) was used as per manufacturer's instructions to isolate plasmid DNA, the DNA was eluted with 30 µl of RNase free water (Qiagen, Crawley, UK). The plasmid DNA was quantified on a Nanodrop spectrophometer (ND-1000, Thermo Fisher Scientific, Wilmington, USA) and diagnostic EcoRI digests consisting of 700-1000 ng of DNA, 2 µl of EcoRI buffer H 10x (Promega, Southampton, UK), 0.2 µl of 10 mg/ml acetylated bovine serum albumin (Promega, Southampton, UK) and 0.5 µl of 20,000 U/ml EcoRI (Promega, Southampton, UK) and RNase free water (Promega, Southampton, UK) to a total reaction volume of 20 µl. The reaction was mixed prior to the addition of the enzyme. The reaction was then incubated in a water bath at 37°C for 2.5 – 3 h. Following incubation the digest reactions were resolved on a 1.5% agarose gel to identify the presence of an insert of the correct size. Positive transformants were subsequently quantified by running 5 µl of plasmid DNA mixed with 2.5 µl Orange G loading dye (Sigma-Aldrich, Gillingham, UK) against 5 µl of 5 ng/µl, 10 ng/µl and 25 ng/µl lambda DNA (Promega, Southampton, UK) again each mixed with 2.5 µl Orange G loading dye (Sigma-Aldrich, Gillingham, UK) on a 1.5% agarose gel containing 2.5 µl of 10 mg/ml ethidium bromide (Sigma-Aldrich Ltd, Gillingham, UK).

(4) Sequencing and Isoform Analysis

Where possible, 500-600 ng of plasmid DNA was submitted for sequencing. DNA sequencing was performed by DNA Sequencing & Services (MRCPPU, College of Life Sciences, University of Dundee, Scotland, www.dnaseq.co.uk) using Applied Biosystems Big-Dye Ver 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer. The Sequencing service provided the M13 primers in order to sequence the insert. These primer sequences are located either side of the insertion site.

Appendix E

Submitted papers

Submitted paper:

"Evidence for a genetic basis of urogenital carcinoma in the wild California sea lion" Submitted to Proceedings of The Royal Society B: Biological Sciences.

Appendix F

Formal presentations of data.

Talks:

"Why is Cancer so Common in California Sea Lions?" - The Marine Mammal Center (TMMC), Sausalito, California, October 2011.

"Investigating the aetiology of urogenital carcinoma in California sea lions (*Zalophus californianus*)" - UK Regional Student Chapter for the Society of Marine Mammals, Sea Mammal Research Unit, St Andrews, February 2012.

"The multifactorial aetiology of urogenital carcinoma in California Sea Lions (*Zalophus californianus*) – A case-control study" - The 61th Wildlife Disease Association (WDA) and 10th European Wildlife Disease Association (EWDA) Joint Conference, Lyon, France, July 2012.

"Investigating the aetiology of urogenital carcinoma in California sea lions" – School of Biology, Post graduate Conference, University of St Andrews, St Andrews, January 2014.

"Investigating the aetiology of urogenital carcinoma in California sea lions" - SMRU lunchtime seminar, University of St Andrews, St Andrews, March 2014

Poster presentations:

"Investigating the aetiology of urogenital carcinoma in the California sea lions (*Zalophus californianus*) - A case-control study" - British Veterinary Zoological Society 50th Anniversary Conference, Cheshire, 2011: Winner of the poster prize.

"Investigating a genetic basis of urogenital cancer in California sea lions (*Zalophus californianus*)" - School of Biology, Post graduate Conference, University of St Andrews, St Andrews, March 2013.

"Investigating a Genetic Basis of Urogenital Cancer in California Sea Lions (*Zalophus californianus*)" - 44th Conference of the International Association of Aquatic Animal Medicine (IAAAM), California, April 2013

"Urogenital cancer in California Sea Lions (*Zalophus californianus*) – A case-control study" - 11th European Wildlife Disease Association conference, Edinburgh, August 2014