UNIVERSITÀ DEGLI STUDI DI SASSARI

CORSO DI DOTTORATO DI RICERCA IN

SCIENZE VETERINARIE

INDIRIZZO: **Patologia e Clinica Animale (XXIX CICLO)**

Biomolecular characterization of *Ovis aries* Papillomavirus 3 in ovine cutaneous squamous cell carcinoma

Docente Guida

Prof. Salvatore Pirino

Il Coordinatore

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Tesi di dottorato della

Dott.ssa Veronica Vitiello

ANNO ACCADEMICO 2015 – 2016

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Università degli Studi di Sassari
Corso di Dottorato di ricerca in
Patologia e Clinica Animale

La presente tesi è stata prodotta durante la frequenza del corso di dottorato in Scienze Veterinarie dell'Università degli Studi di Sassari, a.a. 2015/2016 - XXIX ciclo, con il sostegno di una borsa di studio cofinanziata con le risorse del P.O.R. SARDEGNA F.S.E. 2007-2013 - Obiettivo competitività regionale e occupazione, Asse IV Capitale umano, Linea di Attività l.3.1 "Finanziamento di corsi di dottorato finalizzati alla formazione di capitale umano altamente specializzato, in particolare per i settori dell'ICT, delle nanotecnologie e delle biotecnologie, dell'energia e dello sviluppo sostenibile, dell'agroalimentare e dei materiali tradizionali".

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Abstract

Papillomaviruses (PVs) are associated with benign and malignant tumors of the skin and mucous membranes in humans and animals. Cutaneous squamous cell carcinoma (SCC) is the most common form of skin cancer in sheep and several risk factors, such as PV infection, could increase the risk of tumor development.

In this thesis, we aim to explore the possible involvement of ovine PVs in cutaneous SCC of the sheep, elucidating the pathways involved in viral neoplastic transformation.

Forty SCCs were evaluated by polymerase chain reaction (PCR), in situ hybridization (ISH) and reverse transcription-polymerase chain reaction (RT-PCR) to assess the presence, cellular localization and viral transcriptional activity of ovine PVs. Shotgun proteomic analysis and immunohistochemistry (IHC) were performed to identify and validate infection related proteins.

OaPV3 DNA was detected in 26/40 (65%) SCCs by PCR and in 18/40 (45%) SCCs by ISH. The OaPV3 transcriptional activity was shown in 24/40 (60%) SCCs by RT-PCR. The overexpression of proteins related to the apoptosis and cell death pathways was detected in OaPV3 positive SCCs by the shotgun proteomic analysis. Among the overexpressed proteins, the significant deregulation of the cytokeratin 13 detected in OaPV3 positive SCCs was confirmed by IHC.

The detection of OaPV3 in a high number of SCCs and the identification of infection related proteins, suggest that the viral infection could represent a key factor in the onset of ovine cutaneous SCC.

Thesis organization

The present thesis is organized into three chapters.

The first chapter entitled “General introduction” is a literature review describing the genomic characterization, the life cycle and the pathogenesis of papillomaviruses in human and animals. Particular attention has been focused on benign and malignant lesions occurring in cutaneous and mucosal epithelia associated to species-specific papillomaviruses. Finally, the role of ovine PVs in cutaneous tumor have been introduced.

The second chapter entitled “*Ovis aries* papillomavirus 3 in ovine cutaneous squamous cell carcinoma” explore the ovine papillomaviruses involvement in cutaneous squamous cell carcinoma development, investigating the viral activity in tumors. The manuscript has been submitted and accepted for publication in the Veterinary Pathology Journal.

Finally, the third chapter entitled “Biomarker discovery of *Ovis aries* papillomavirus 3 in ovine cutaneous squamous cell carcinoma” contains an investigation of proteins related to the viral infection in cutaneous squamous cell carcinoma, in order to identify putative biomarkers of OaPV3 in tumor.

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Chapter I

Chapter I

General Introduction

1.1 Tumors of sheep

The ovine species, one of the most numerous agricultural species worldwide, is particularly important in Europe, in which the presence of around one hundred millions of sheep has been estimated (European Commission, COM (2013)). Sheep farming for milk, meat and wool represents a leading productive reality in several countries, making this species of particular interest in the livestock sector.

In the last few decades, the application of more effective drugs and vaccines, as well as the improvements in diagnostic technologies, resulted in a general reduction of livestock diseases (Thornton, 2010). Nevertheless, several pathological processes are frequently reported in the ovine species, especially regarding the genital system, since this animals are used for reproductive purpose and for milk and meat production. Neoplastic disorders in others farm animals, such as bovine, have been deeply investigated, leading to identify specific predisposing factor related to cancer development. Conversely, tumors are less studied in sheep, although the incidence of neoplasms appears to be low in this species.

The low incidence of ovine tumors was firstly reported by Williams in the first decade of the twentieth century and then confirmed by several studies investigating tumors of farm animals. Hodgson, Crocker and Feldman reported the observation of only few ovine tumors in large observational studies in the abattoir, mostly of epithelial origin (Hodgson, 1903; Crocker, 1919; Feldman, 1931). Furthermore, Feldman identified the gastrointestinal tract of the sheep as an uncommon site for the tumor development (Feldman, 1931). Moreover, more up-to-date studies suggest a segmental insusceptibility restricted to the upper gastrointestinal tract of the sheep, in which only two reports described fibropapillomas and squamous cell carcinoma in the rumen and in

the omasum, respectively (Doige, 1983; Norval *et al.,* 1985). Conversely, the small intestine adenocarcinoma is particularly reported in sheep of New Zeeland, Scotland, and Australia, where the involvement of dietary carcinogens in tumor development appears possible, though not fully demonstrated (Evans, 1968; Simpson and Jolly, 1974). Considering the limited occurrence of this tumor in the mentioned countries, only few studies investigated the etiology of the small intestine adenocarcinoma in the ovine species. Tumors affecting the ovine respiratory system have been instead thoroughly investigated, since a viral etiology has been suspected from the 19th century (Palmarini and Fun, 2001). Similarly, neoplasms of the hematopoietic system have been deeply studied for the high frequency of tumors during meat examination in the abattoirs. Specific retrovirus associated tumors have been identified in both respiratory and hematopoietic systems of the sheep, leading to perform several studies investigating the mechanisms of viral pathogenesis and transmission in breeds (Griffiths *et al.*, 2010; Aida *et al.*, 2013). Nevertheless, the incidence of these tumors, as well as the viral transmission rates, are very difficult to estimate since most of the infected animals remain asymptomatic (Caporale *et al.*, 2006; Gillet *et al.*, 2007). Based on these consideration, skin tumors are considered as the most frequently reported neoplasms of the sheep (Hassanein and Mahmoud, 2009; Ahmed and Hassanein, 2012). Although multiple factors, including the ultraviolet radiation and the Papillomavirus infection, seem to play an important role in cutaneous tumors development, the etiology of these neoplasms is not still clear (Mendez *et al.*, 1997; Alberti *et al.*, 2010).

The study of several type of ovine tumors may be an on-going motif of research for understanding cancer pathogenesis in human, as recently proposed for the lung neoplasms by Youssef and colleagues (2015). Furthermore, as suggested by Palmarini and Fan (2001), the study of molecular mechanisms occurring in virus induced ovine

neoplasms is an important tool to investigate human tumors. Based on these considerations, a detailed study of most commonly occurring tumors in sheep, as well as the investigation of the molecular pathogenesis, may provide advances in diagnosis and treatment of several type of human tumors, and will impact on the welfare of farm animals.

1.2 Virus-induced tumors in sheep

Oncogenic viruses are important pathogens in ovine species, able to promote malignant tumors development by a multistage process (Teklemarian *et al.*, 2015). Oncogenic virus are classified into different virus families, including both DNA and RNA viruses causing several economic loss in animal husbandry (Efird *et al.*, 2014).

As mentioned above, members of the *Retroviridae* family represent the most important oncogenic RNA virus reported in both respiratory and hematopoietic systems of the sheep. However, also the *Papillomaviridae* family seems to play an important role in the etiology of ovine skin tumors, although the viral activity in malignant lesions remains unclear.

1.2.1 Retrovirus

The *Retroviridae* family comprise a large group of enveloped RNA viruses that carry two identical copies of genomic RNA in the virion (Coffin *et al.*, 1997; Fan and Johnson, 2011).

Retrovirus have relatively simple genomes of 8-12 Kb in length containing three major coding domains, named *gag*, *pol* and *env* (Fan and Johnson, 2011). In particular, *gag* directs the synthesis of internal virion proteins, *pol* contains the information for the reverse transcriptase and integrase enzymes, and *env* contains transmembrane components of the viral envelope protein. An additional smaller, coding domain is *pro*, encoding the virion protease (Coffin *et al.*, 1997).

Retroviruses are classified as acute transforming retroviruses and nonacute retroviruses, based on the genome organization. Acute transforming retroviruses induce tumors of polyclonal origin rapidly by a process of acquisition and expression of cellular proto-

oncogenes, named oncogene capture (Liu and Miller, 2007). During this process, the coding sequences for the viral oncogene typically are altered in comparison to the cellular proto-oncogene. As a result, the expression of viral oncogenes cause uncontrolled stimulation of cell growth (Fan and Johnson, 2011). Interestingly, some or all of the genes required for viral replication are deleted when the cellular oncogene is captured. For this reasons, acute retrovirus are often replication-defective (Coffin *et al.*, 1997).

Conversely, nonacute retroviruses not carry oncogenes and induce tumors slowly by a process of proviral insertional mutagenesis, in wich the integration of the proviral DNA occurs near normal cellular proto-oncogenes (Liu and Miller, 2007). The insertion of the provirus introduces a strong promoter and enhancer sequences into the gene locus, leading thus to an altered gene expression of cellular proto-oncogenes (Butel, 2000). During a normal retroviral infection, the integration of proviral DNA is a random process with respect to the host chromosomal DNA. However, in tumors induced by nonacute retrovirus, it has been found that there are common chromosomal sites of insertion resulting in transcriptional over-expression of the adjacent proto-oncogene (Fan and Johnson, 2011).

The first step in the retrovirus replicative cycle is the adsorption of viral particle to the surface of the target cells. This process occurs by the interaction of the viral proteins with the corresponding cell-entry receptors on the host cell surface and allows the fusion of the viral envelope with the host cell membrane. The viral core is thus injected into the cytoplasm, where the viral RNA is reverse transcribed into linear double-stranded DNA by the virion-packaged reverse transcriptase (Nisole and Saib, 2004; Liu and Miller, 2007). The viral DNA enters the nucleus and integrates into the host genome, leading to the formation of the provirus (Hofacre and Fan, 2010). After

integration, the viral RNA is transcribed by the cellular RNA polymerase II and then processed like normal cellular mRNA. Viral RNA is subsequently exported to the cytoplasm and then translated to proteins by cellular protein synthesis machinery. Finally, virions are assembled into immature particles in the perinuclear compartment and subsequently released from the cell, leading to their maturation in infectious virus particles (Hofacre and Fan, 2010).

Retrovirus are classified into two subfamilies, named Orthoretrovirinae and Spumaretrovirinae. The subfamily Orthoretrovirinae is further subdivided into six *genera*, Alpharetrovirus, Betaretrovirus, Gammaretrovirus, Deltaretrovirus, Epsilonretrovirus, and Lentivirus, whereas the subfamily Spumaretrovirinae includes only the single genus of Spumavirus (Fig 1.8). Unlike the others genus, Lentivirus and Spumavirus are not oncogenic (Maclachlan and Dubovi, 2016).

1.2.1.1 Ovine betaretrovirus

The Betaretrovirus *genus* included enzootic nasal tumor virus (ENTV) and Jaagsiekte sheep retrovirus (JSRV), two closely related retroviruses that cause naturally occurring neoplasms of the respiratory tract of the sheep (Alberti *et al.*, 2002; Liu *et al.*, 2003). ENTV is able to transform secretory epithelial cells of the nasal mucosa, leading to enzootic nasal adenocarcinoma (ENA) (Yu *et al.*, 2011). The clinical signs of this tumor depend on the size, growth rate and extent of the tumor although frequently affected sheep have profuse seromucinous nasal exudate, dyspnea, stertorous brething, and coughing. (McKinnon *et al.*, 1982). Similarly, JSRV transforms secretory epithelial cells (Clara cells and type II pneumocytes) of the distal lung leading to ovine pulmonary adenocarcinoma (OPA), a tumor reported worldwide, except in Australia and New Zeland (Wilson, 2017). Iceland is now free from OPA following a rigorous slaughter

policy in the 1950s (Griffiths *et al.*, 2010). Viral transmission of JSRV occurs through the aerosol route by inhalation of infected respiratory secretions, although the virus may also be transmitted via colostrum and milk. The disease has a long incubation period before clinical signs are evident, leading to a high prevalence in infected flocks. Clinically affected sheep show a progressive respiratory distress that worsen with increasing size of the lesions (Palmarini and Fun, 2001).

Both of these viruses are unique among retroviruses in the transformation process of secretory epithelial cells of the respiratory tract. JSRV and ENTV have the canonical genomic organization of retrovirus, with the four common retroviral genes encoding essential viral proteins. The long terminal repeat (LTR) sequence located in the viral genome contains the viral promoter and enhancer elements that specifically interact with the cellular transcription machinery, driving the viral expression. An additional open reading frame, named orf-x, is also present in JSRV genome and is of undefined function (Griffiths *et al.*, 2010). The examination of the JSRV and ENTV genome sequences revealed no apparent oncogene, leading to classified these viruses as nonacute retrovirus (Liu *et al.*, 2003), although both JSRV and ENTV do not express a hostderived or auxiliary oncogene (Leroux *et al.*, 2007). For all these reasons, the mechanism of ovine betaretrovirus inducing malignant transformation have been thoroughly investigated. Unlike classical retroviral mechanism of insertional activation and host oncogenes capture, a distinct process of JSRV and ENTV oncogenesis has been reported (Maeda *et al.*, 2001; Wooton *et al.*, 2005). In particular, it has been demonstrated that the env proteins of both viruses are able to transform primary and established cell lines in vitro by a specific host cells interaction LTR sequence-mediated, leading to consider the env codified products as responsible of oncogenesis in animals (Maeda *et al.*, 2001; Alberti *et al.*, 2002; Liu *et al.*, 2003).

1.2.1.2 Ovine Deltaretrovirus

In the Deltaretrovirus genus is included the bovine leukemia virus (BLV), a highly cell-associated retrovirus able to produce a chronic infection in its natural hosts (cattle and water buffalo) and also in sheep and goats. In sheep, BLV is associated with the lymphoma development, a widely disseminated tumor arising in lymphoid tissues outside of the bone marrow (Valli *et al.*, 2017). Ovine lymphoma is similar to the calf or juvenile type of bovine lymphoma, but without leukemia and marrow involvement. Infection is transmitted horizontally by direct contact and, once established, is characterized by the development of circulating antibodies that increase with the number of viral antigens recognized. Unlike cattle, BLV does not produce persistent lymphocytosis in sheep but the virus is able to induce the tumorous state without prior mutations in the p53 tumor suppressor genes (Maxie, 2007).

BLV genome contains the structural gag, pol and env genes required for the synthesis of the viral particles and an additional X region encoding for two structural protein, named Tax and Rex. Tax is a transactivator protein and is the major factor required for viral replication and pathogenesis. Tax is able to recruit cellular transcription factors forming a multiprotein complex that drives gene expression. Rex is a regulator of expression protein that is required for the accumulation of genomic and singly-spliced env RNA (Palmarini *et al.*, 2000; Gillet *et al.*, 2007). The primary target cell of BLV is a B lymphocyte, which expresses the immunoglobulin M on the surface. As described above, persistent lymphocytosis do not occurs in sheep and the tumor develop rapidly (Mirsky *et al.*, 1996).

1.2.2 Papillomavirus

1.2.2.1 Genome

PVs are small, non-enveloped, circular double stranded DNA virus, with a genome ranged from 6953 bp [Chelonia mydas papillomavirus type 1 (CmPV1)] to 8607 bp [Canine papillomavirus type 1 (CPV1)] in length (Van Doorslaer, 2013). The genome of all PVs is divided into three region, separated by two polyadenilation sites: the long control region (LCR), containing the control elements for replication and transcription of the viral DNA, and two regions containing the open reading frames (ORF) corresponding to early (E) and late (L) genes (Fig 1.1).

The early region occupies over 50% of the virus genome and usually contains six open reading frames (ORF), named E1, E2, E4, E5, E6 and E7. The proteins encoded by the early ORF have regulatory functions in replication and transcription of the PV genome, in cell cycle, cell signaling, in the apoptosis control and in structural modification of the infected cell. In particular, E1 and E2 proteins modulate transcription and replication, whereas E5, E6 and E7 proteins modulate the transformation process and represent the main oncoproteins in some PV types.

The late region covers almost 40% of the virus genome and contains two ORF, L1 and L2. The products encoded are the major and the minor protein capsid of the virus required for transmission, spread and survival in the environment. The LCR region, covering about the 10% of the genome, has no protein-coding function, but represents the origin of replication and contains multiple transcription factor binding sites as well as the control elements for replication and transcription of the viral DNA (Zheng and Baker, 2006; Graham, 2010).

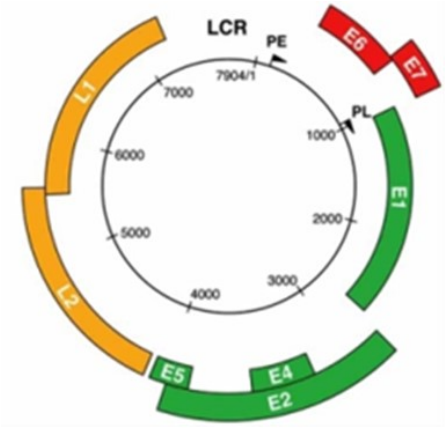

Figure 1.1 Typical organization of the PV genome (Doorbar *et al.*, 2015).

1.2.2.2 Viral early proteins

In the early region, the E1 gene codifies for a hexameric DNA helicase E1 that represents the only protein with enzymatic activity codified by the PV. This helicase orchestrates the initiation and catalysis of viral DNA synthesis, maintaining constant level of episomes in cells and promoting amplification of the viral genome during the productive phase of the viral life cycle. In addition, E1 protein interacts with specific host factors to promote the assembly of the replisome, necessary for bidirectional replication of the viral genome (Bergvall *et al.*, 2013).

The E2 gene codifies for a multifunctional protein that represents the main transcriptional regulators of the PVs, able to activate or repress the transcriptional process recruiting factors that manipulate cellular chromatin processes. The E2 protein participates in all phases of the PVs life cycle, orchestrating the replication, the partitioning and the amplification processes. In the replication of the viral DNA, the E2 protein promotes and supports the initiation process by loading the E1 helicase onto the replication origin. Furthermore, in the partitioning process, the E2 protein is required for the maintenance of the genome in episomale form and to facilitate the retention, the maintenance and the partitioning of the viral genome. Finally, this protein is required

for vegetative viral DNA amplification and some study highlighted an E2 protein overexpression in that late phase of the infection (McBride, 2013). The E4 gene product, obtained from a spliced mRNA, is an E1^E4 fusion protein that includes the initiation codon and first few amino acids from the E1 ORF and the remaining of the E4 gene. Interesting, the expression of the fusion protein and her presence in the cytoplasm of infected cells is clearly visible in histological sections as granules, even just with the Haematoxylin and Eosin stain. These inclusion granules are tipically noticeable in the mid epithelial layers and are considered by the pathologist as a major contributor to the cytopathic effect of the PVs. The E1^E4 protein is able to facilitate and support viral genome amplification and to regulate the expression of the late genes. The E4 is also involved in the stimulation of cell cycle progression and in the interaction with cellular keratin networks, causing the collapse and promoting the escape of the mature virions from the cornified cells (Conway and Meyers, 2009; Doorbar, 2013). The E5 gene, not present in all PVs, codified for the smallest PVs oncoprotein that represent the major oncogene in bovine PV (DiMaio and Mattoon, 2001). While the oncogenic activities of E6 and E7 are well characterized, the role of E5 is still rather nebulous. E5 interacts with a number of cellular proteins and these interactions are deemed important for the biological activity of the protein in cell transformation and evasion of the immune response (Venuti *et al.*, 2011). The product of the E6 gene is a protein, containing two zinc-like fingers, considered as one of the most important PVs oncoproteins. In fact, the E6 protein is able to modulate the transcription of p53-dependent genes interacting with the core region of p53, inducing the degradation gene, or by different mechanism that include an aberrant p53 localization and post-translational modifications of the p53 protein. A consequence of the E6 degrading or blocking p53 function is the inhibition of the apoptotic signals that can’t contribute to eliminate the abnormal cells. Moreover,

different study have showed that E6 is able also to disrupt some pathways that are triggered in presence of different stresses, preventing the cell death (Howie *et al.*, 2009). Similarly to the E6 gene, the E7 gene, not present in all PVs types, codifies for another important protein, the E7, with oncogenic activities, contributing to virus-induced cellular transformation. The E7 protein specifically binds to a region of the Rb protein, usually named "pocket domain" and essential for its tumor suppressor function, and breaks the interaction between Rb and E2F-family transcription factors. The consequences of this bind are both the repression of the replication enzyme genes expression and of the tumor suppression function of Rb (Yim *et al.*, 2005).

1.2.2.3 Viral late proteins

The L1 and L2 genes codified for two proteins with structural function, expressed in the late phase of the life cycle. L1 and L2 are respectively the major and the minor viral capsid protein and the interaction of both these proteins allow the viral capsid assembly. In particular, the transposition first of the L2 and then of the L1 protein from the cytoplasm into the nucleus of infected cells, allows the self-assembly of the virus capsid. PV capsid is composed of 72 pentameric capsomeres arranged in a T7 icosahedral lattice. Each capsomere contains five monomers of the 55-kDa L1 protein and ca. 12 copies of the 74-kDa L2 protein, probably associated with 12 pentavalent capsomeres. Complexively the capsid shows an elegant icosahedral surface (Fig. 1.2).

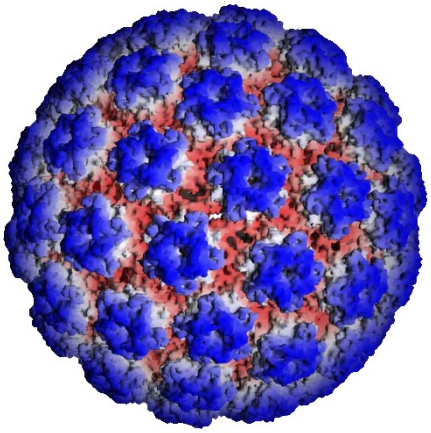

Fig 1.2 Atomic model of the Papillomavirus caspid (Modis *et al.*, 2002).

1.2.2.4 Life cycle

The majority of PVs share a tropism for the basal layers of the stratified epithelium and replicate exclusively in epithelial cells, with a life cycle tightly regulated by cellular differentiation (Doorbar, 2005; Mistry *et al.*, 2008; Graham, 2010). Initial infection requires epithelial wounding to allow virus access to the basal layer of epithelium (Fig 1.3) (Conway and Meyers, 2009). The virus infect an epithelial stem cell of the basal layer, that continuously divide and replenish epithelial cells desquamated (Egawa, 2003; Doorbar, 2005). Virions are first adsorbed in basal cells by interaction with heparan sulfate, and then internalized into the nucleus by clathrin-coated endocytic vesicles or the clathrin independent caveolae system, which produces uncoated vesicles (Day *et al.*, 2003). Several structural changes, as uncoating and cellular sorting are necessary to ensure the correct transfer of the viral genome to the nucleus (Doorbar *et al.*, 2012).

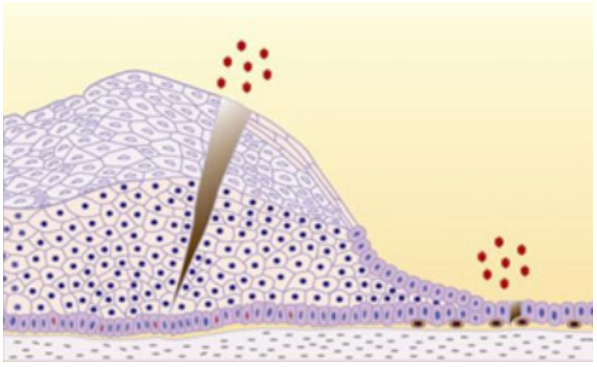

Fig 1.3 Infection and access of viral genome to the basal cells (Doorbar, 2012).

In the nucleus of infected cells, viral infection is followed by an initial phase of genome maintenance, in which the viral DNA is maintained as an episome at low copy number (Doorbar, 2005). The pattern of viral gene expression in this phase of the life cycle is not well defined (Doorbar, 2015), nevertheless different studies reported the expression of E1 and E2 genes that are required for the episomal maintenance and the cellular polymerases recruitment (Conway and Meyers, 2009). These two proteins form a complex with the viral origin of replication and recruit cellular polymerases and accessory proteins to facilitate replication. The expression of E1 and E2 proteins also regulates the transcription of E6 and E7 genes, driving the cell proliferation in basal and parabasal cell layers and allowing the viral life cycle in the proliferative phase (Fig 1.4) (Conway and Meyers, 2009). Basal epithelial cells migrate into the suprabasal layers in order to undergo terminal differentiation. E6 and E7 proteins stimulate cell cycle re-entry in the mid-epithelial layers by the interaction with regulators, as p53 and the retinoblastoma protein (pRb), respectively. In particular, the E7 interaction with the pRb protein allows the cellular S-phase entry and leads the expression of proteins necessary for DNA replication, while E6-p53 interaction prevent the apoptosis process (Doorbar, 2005). Interestingly, it appears that the ability of E7 to drive cellular proliferation could be inhibited in some cells, depending on the levels of the p21 and p27 cyclin-dependent

kinase inhibitors. The ability of E7 to drive cells through mitosis in differentiating epithelium may be limited to those cells which express p21and p27 at low level or which express sufficient E7 to overcome the block to cell-cycle progression (Doorbar, 2006).

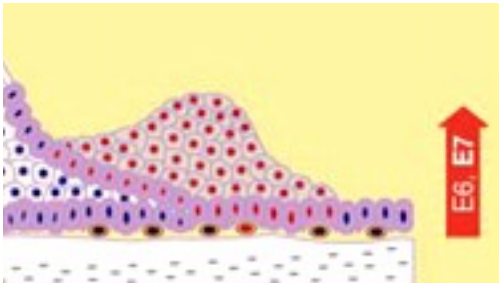

Fig 1.4 E6 and E7 proteins in basal and parabasal epithelial layers (Doorbar, 2012).

Although cell proliferation is required for lesion formation and the maintenance of viral episomes in basal and parabasal layers, PVs must eventually amplify and package their genomes in infect cells migrating towards the epithelial surface. In the phase of genome amplification, an up-regulation of the late promoter leads an increased expression of proteins involved in viral DNA replication (Doorbar, 2005). The E2 codified protein specifically binds an upstream regulatory region necessary for viral DNA replication and recruits the E1 DNA helicase to the viral origin of replication. The resulting E1-E2 initiation complex allows the replication of viral genomes and proceed independently of cellular DNA synthesis, leading to an increase in viral copy number in the upper epithelial layers (Doorbar, 2005). As proposed by Middleton and colleagues (2003), the newly replicated genomes could represent the templates for the further expression of E1 and E2, allowing an additional amplification of viral genomes (Middleton *et al.*, 2003). Once viral genome amplification is completed, the two structural proteins L1 and L2 are expressed in the upper epithelial layers in order to package the amplified genome into

the capsid (Fig 1.5). The minor coat protein L2 is able to enhance genome packaging in cells expressing E4, inducing the accumulation of PML bodies into the nucleus during virus assembly. Conversely, the major capsid protein L1 is expressed after L2 and provides the assembly of the icosahedral capsid in the nucleus with the final production of extremely stable infectious virions in the upper layers of the epithelium (Doorbar, 2012; Doorbar, 2015). In the cornified envelope, E1 and E4 proteins interact with cellular keratin networks, causing their collapse, thus allowing mature virions to escape from the cornified cells (Conway *et al.*, 2009). The extracellular survival of Papillomaviruses is enhances by the desiccation-resistance, facilitating the re-infection in a new host (Doorbar, 2005).

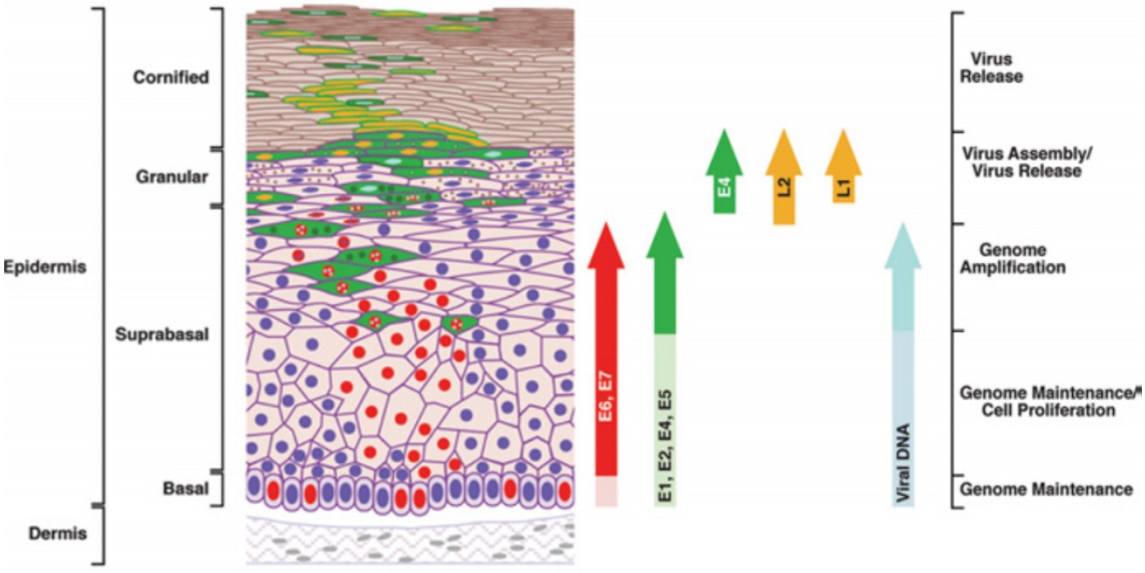

Fig 1.5 The papillomavirus life cycle: the key events that occur following infection are shown diagrammatically on the left. The epidermis is shown in color with the underlying dermis being shown in grey. The different cell layers present in the epithelium are indicated on the left. Cells in the epidermis expressing cell cycle markers are shown with red nuclei. (Middleton *et al.*, 2003)

1.2.2.5 Latent phase of Papillomaviruses

Viral infection can have several outcomes. In the acute phase, the infection is followed by the complete elimination of the virus from the host, while in a chronic or persistent phase, the infection may result with long-term carriage of the virus. Nevertheless, a state of viral latency without clinical signs of disease may develop following infection (Maglennon and Doorbar, 2012).

The latent stage of the viral life cycle has been reported in many viruses, including Papillomavirus.

First evidences of this life stage in humans include the reappearance of papillomas in the larynx of children and the detection of HPV11 in these lesions, as well as the rapid onset of genital and cutaneous papillomas in immunosuppressed transplant patients (Steinberg *et al.*, 1983; Penn, 1986; de Villiers *et al.*, 1997). Additionally, it appears that some economically important diseases may also be associated with latent infection in animal species, as in bovine and horses (Maglennon and Doorbar, 2012). In these cases, two bovine Papillomavirus (BPV), named BPV1 and BPV2, are associated with both urinary bladder cancer and equine sarcoids in cow and horses, respectively (Borzacchiello *et al.*, 2003; Chambers *et al.*, 2003). The frequent detection of PVs in normal skin of horses affected by equine sarcoids and the development of lesion at sites of wounding, suggests a possible viral reaction (Bogaert *et al.*, 2008). Similarly, the detection of BPV4 DNA in normal bovine bladder mucosa has suggested the latent viral presence and the possible reactivation in presence of co-carcinogenic factor, as bracken fern (Carr *et al.*, 2001).

Different study investigating the latent stage of the cottontail rabbit papillomavirus (CRPV) have reported this PV types as the best model system (Stubenrauch *et al.*, 1999). Infection of domestic rabbits with CRPV in multiple sites induces papillomas

over a period of days (Schmitt *et al.*, 1996). Interestingly, papillomas resulted in a low number of the infected sites, although in the remaining normal skin viral DNA can be detected at low copy number (Stubenrauch *et al.*, 1999). These observations lead to considered the host immune system as the regulator of the viral latent state that, once activated, is able to restrict the PV infection in basal cells and leads altered levels of viral activity in which only very few cells are able to support the productive viral cycle (Doorbar, 2005). Interestingly, different experimental studies revealed the presence of CD8+ T cells and leukocytes into the epithelium and demonstrated that virus-infected cells are recognized by infiltrating CD8+ cells with an effect strongly amplified by TNF-α-secreting leukocytes (Selvakumar *et al.*, 1997). In the presence of a strong immune response, papillomas can undergo spontaneous regression, although this process does not result in elimination of all infected cells. For this reason, a subsequent virus reactivation may occur upon suppression of the immune system. Nevertheless, latent viral presence in infected cells includes the maintenance of the viral genome as an episome as well as low level of viral transcripts (E1 and E2) and proteins production (Maglennon and Doorbar, 2012). For these reasons, the detection of viral DNA and RNA in a latent stage may be very difficult by conventional methods, as PCR and RT-PCR, and in some case the experimental results are conflicting (Maran *et al.*, 1995).

1.2.2.6 Classification

Papillomaviridae is a heterogeneous family of highly species-specific virus able to infect mammals, birds and reptile species (Aldabagh *et al.*, 2013). Originally, PVs had been included in the Papovaviridae family, together with the Polyomavirus. This was based on similar, nonenveloped capsids and the common circular double-stranded DNA genomes. Subsequently, the International Committee on the Taxonomy of Viruses

(ICTV) has recognized these groups as two distinct families of viruses, *Papillomaviridae* and *Polyomaviridae*, due to the different genome size and organization (de Villiers *et al.*, 2004).

PVs genome shows a remarkable conservation due to the presence of a common core of genes reported in all PVs characterized to date and named E1, E2, L1 and L2 genes (Rector and Van Ranst, 2013). In particular, the L1 gene represents the most conserved region and is currently used for the construction of phylogenetic trees and for the identification of new PV types. The current classification, described by de Villiers and colleagues in 2004 and recently expanded by Rector and Van Ranst in 2013, is based on the difference of the L1 nucleotide sequence and aim to establish three different levels for each PV:

- ✓ genera: defined by more than 60% nucleotide sequence dissimilarity
- ✓ species: established by nucleotide identity between 60% and 70%
- ✓ types: defined by nucleotide identity between 71% and 89%

In addition, subtypes of PV types are defined by homology differences of 2-10% and variants as having homology differences by 1-2 % (de Villiers *et al.*, 2004). PVs within a genus often demonstrate similar host, location, and behavioral characteristics (Munday, 2014a), whereas PVs within the same species have common biological and pathological properties (de Villiers *et al.*, 2004). For this reason, the application of this classification system allows to generate highly informative phylogenetic trees for the human and animal PVs described.

The nomenclature to identify a specific genus is based on the Greek alphabet with the prefix “dyo”, except for the human Alpha-, Beta- and Gamma-PV genera that include the most common and medically important PVs. In addition, the animal PV types nomenclature is based on the scientific name of the host, using the host genus and

species designation, followed by a different number for each PV type (i.e. FdPV1 for Felis domesticus PV type 1) (de Villiers *et al.*, 2004; Bernard *et al.*, 2010).

1.2.2.7 Diagnosis of Papillomavirus infection

The traditional methods of viral diagnosis such as electron microscopy, cell culture, and certain immunological methods are not suitable for all PV types detection (Dixit *et al.*, 2011). Additionally, several methods have a limited sensitivity and specificity, giving different false negative rates. For this purpose, novel strategies have been performed in order to increase the accuracy of viral detection.

To date, the presence of PVs can be inferred from morphological, serological and clinical findings, although PV diagnosis relies on molecular biology techniques, allowing the accurate viral detection and typing. Nucleic acid-hybridization assays and nucleic-acid amplification are the most common methods available (Abreu *et al.*, 2012).

Nucleic acid hybridization assays include techniques such as Southern blotting, in situ hybridization and dot-blot hybridization. Nucleic acid hybridization assays are based on the hybridization of the target PV-DNA to specific labeled DNA probes. These techniques generated high-quality information, although show a low sensitivity and need of a large amounts of purified DNA (Abreu *et al.*, 2012).

Nucleic-acid amplification includes several high sensitive techniques, such as polymerase chain reaction (PCR), real-time PCR and reverse transcription-PCR (RT-PCR). PCR is the most widely used amplification method, using a thermo-cycling process and employing specific oligonucleotide primers to amplify DNA in presence of a thermostable DNA polymerase. Two different PCR approaches for detection of PV-DNA have been performed, including type specific or broad-spectrum methods. Type specific PCR is based on specific primers designed to amplify exclusively a single PV

genotype. This method is expensive and the specificity of each primer set should be validated (Molijn *et al.*, 2005). Conversely, broad-spectrum PCR includes consensus or general PCR primers, targeting a conserved region of PV genome. Since E1 and L1 regions represent the most conserved regions of the PV genome, several consensus PCR primer sets are aimed at this region (Zaravinos *et al.*, 2009). Nevertheless, this method is strictly dependent on the choice of primers and on the size of the PCR products, influencing the efficiency of the PCR reactions (Abreu *et al.*, 2012). Results of PCR reaction can be easily detected by standard agarose gel electrophoresis. However, subsequent sequence-specific analysis increase both the sensitivity of the assay. Several methods have been developed for this purpose, including direct sequence analysis of PCR products and restriction fragment length polymorphism (RFLP). The direct sequence analysis is useful in the PV genotype determination and can be performed by two methods. In one case, the sequence homology is investigated by different software on databases, while in the other case a phylogenetic analyses is performed in a multi-sequence alignment with a set of known PV sequences (Molijn *et al.*, 2005).

The RFLP method is a simple technique in which the amplified DNA is digested by restriction enzymes, resulting in DNA fragments of various lengths. The PCR-RFLP show a good discriminatory capacity and is easier and less expensive than sequencing (Abreu *et al.*, 2012).

Real-time PCR is another amplification method that can be used to detect PV-DNA. Real-time PCR is a quantitative analysis, able to detect low amounts of PV DNA that are compared with a standard reference template (Piana *et al.*, 2009). This technique is based on type-specific PCR primers combined with fluorescent probes for DNA real-time detection. Additionally, multiplexing PCRs using multiple primers to allow amplification of multiple templates within a single reaction have also been developed

(Mackay *et al.*, 2002). Although real-time PCR is an expensive techniques, this method represents a reliable, sensitive, and specific diagnostic tool for detection and genotyping of targeted PV genotypes (Abreu *et al.*, 2012). The use of this method is particularly useful in human PV infection in which a large number of PV genotypes are involved in cervical cancer. In this case the differentiation and the quantification of PV genotypes are required in order to clarify the disease risks and monitor the progression of the PV infection (Liao *et al.*, 2013).

RT-PCR allows the detection of specific viral RNA by a reverse transcription step before PCR amplification. This method provide accurate information on viral gene expression in cells or tissues by using gene specific primers (Molijn *et al.*, 2005). RT-PCR can be carried out in one-step or two-step methods. In one step RT-PCR, reverse transcription and PCR are performed sequentially in the same reaction, while in two step method cDNA synthesis is performed first and then PCR reaction is carried out (Santos *et al.*, 2004).

1.3 Human Papillomavirus

To date, HPVs are the most intensively studied group, with more than 140 different viral types identified and classified within 5 genera (alpha, beta gamma, mu and nu). The completely sequenced genomes are reported as Reference Genomes of HPV in the Papillomavirus Episteme (http://pave.niaid.nih.gov).

HPVs are able to infect basal epithelial cells of the skin or mucosal tissue and are consequently categorized as cutaneous or mucosal type, based on their tropism. Cutaneous types target mostly the skin of the hands and feet, leading to the development of warts (plantar warts, common warts and flat warts). Cutaneous infection is transmitted by direct contact with an infected tissue or indirectly by contact with contaminated objects. Lesions usually regress spontaneously within 1 to 5 years, although may undergo malignant transformation into squamous cell carcinoma. This condition is particularly observed in patients affected by epidermodysplasia verruciformis, a rare, autosomal recessive genetic disorder of the immune system manifested by an increased susceptibility to cutaneous HPV infection.

Mucosal types infect cells of mouth, throat, respiratory tract, and anogenital epithelium (Burd, 2003). About one third of reported HPV infect epithelial cells in the genital tract, causing low or high grade cervical lesions. Based on their pathogenicity, mucosal PV types have been classified into two categories, namely low risk and high risk. Low risk HPV, such as HPV 6 and 11, are mainly found in benign lesions characterized by a limited tendency to malignant progression, as genital warts. Conversely, high-risk types, such as HPV 16 and 18, are associated with the occurrence of pre-malignant and malignant cervical lesions (Muñoz *et al.*, 2003).

1.3.1 Human Papillomavirus and cervical cancer

The link between HPV and cervical cancer was first demonstrated in the early 1980s by Harold zur Hausen, a German virologist. In 1996, the World Health Association recognized HPV as an important cause of cervical cancer, implicated in more than 99% of cervical squamous cell carcinoma cases worldwide. To date, the association between HPV and cervical cancer it has been thoroughly investigated, leading to identify a persistent infection by high risk HPVs as the necessary cause of cervical cancer (Burd, 2003).

Cervical cancer is the second most common cancer in women worldwide and is the most frequent cancer in many developing countries. The presence of 470.000 new cases of cervical cancer per year has been estimated worldwide, leading to considered HPVs as one of the most common causes of sexually transmitted disease in both men and women worldwide (Gómez and Santos, 2007). Viral infection is particularly prevalence in young adults at the beginning of their sexual activity, with a subsequent decline with increasing age due to the development of an immune response against the virus and the reduction of sexual acitivity (Chan *et al.*, 2010). The number of sexual partners is the risk factor more consistently associated with genital HPV infection, although other indicators of sexual behavior and reproductive activities, heredity, immune and nutritional status can contribute to the development of cancer (Fernandes and de Medeiros Fernandes, 2012).

Cervical cancer arises in the cervical transformation zone, located between the squamous epithelium of the ectocervix and the columnar epithelium of endocervix. Basal cells in the transformation zone have the ability to differentiate, the most important property required for virion production (Fernandes and de Medeiros Fernandes, 2012). Furthermore, the presence of hormones, such as estrogen and

progesterone, can help HPV infection. It has been estimated that the HPV life cycle takes two or three weeks, the time necessary for a cervical cell to migrate from the basal to most superficial layers of the epithelium and undergo to maturation and senescence process. However, the viral expression of some high risk HPV acts as a negative regulators of the cell cycle and the infected cells do not undergo to the maturation process. In particular, the expression of E6 and E7 viral oncogenes leads specific interactions with products of tumor suppressor genes. The E6 protein targets TP53 preventing apoptosis and facilitating the replication of transformed cells. Conversely, E7 protein contributes to the oncogenesis process by the interaction with members of the retinoblastoma family, the so-called pocket proteins. E7 targets these proteins, inhibiting their ability and promoting DNA synthesis. Furthermore, the E5 protein of high risk HPV works with E6 and E7 proteins to drive cellular proliferation, representing a weak cofactor in development of malignancy (Crosbie *et al.*, 2013). The process of oncogenesis includes a gradually progression from mild cervical intraephitelial neoplasia (CIN) to severe degree of neoplasia (CIN 2 and CIN 3) and finally to invasive cancer. Mild dysplasia is associated with continued viral replication, although most of these lesions usually spontaneously regress. Subsequently, the conversion from an episomal to an integrated form of the viral genome, as well as the expression of the E6 and E7 viral oncogenes, lead to the progression to high grade lesions (CIN 2 and CIN 3) and ultimately invasive cancer (Gómez and Santos, 2007). Cancer development depends not only on efficient negative regulation of the cell cycle, but also on a sophisticated system of immune evasion developed by high risk PVs, in which the E6 and E7 proteins play a central role. The expression of these proteins inhibits the transcription of toll like receptor 9 and reduces the expression of key components of the peptide processing and presentation pathway, preventing the

activation of specific cytotoxic T lymphocytes. Furthermore, the expression of E6 and E7 proteins inhibit the interferon synthesis and reduce the expression of proinflammatory signals. Despite this immune evasion mechanism, most PV infections are cleared in few months by the involvement of the cell-mediated immunity and the development of a specific antibody response to the major viral capsid protein (Crosbie *et al.*, 2013).

1.3.2 Vaccine

An effective vaccination against PV represents an important prophylactic tool, particularly in human, in which different PV types are associated to cervical cancer (Hung *et al.*, 2008). Although the exposure of immune system to PV antigens is limited, both innate and adaptive immunity are engaged in viral infection. For this reason, both prophylactic and therapeutic PV vaccines have been developed in human. Prophylactic vaccines are successful used at preventing healthy patients from acquiring PV infections (Frazer, 2009; Nayereh and Khadem, 2012). The aim of these vaccines is to generate strong type-specific immune responses against the most prevalent PV types. The immune response is able to induce the neutralizing antibodies as well as to recall the specific memory cells for future viral infections (Nayereh and Khadem, 2012). In particular, natural PV infections induce low antibody concentrations in human, in which the viral L1 proteins show self-assemble properties, producing virus-like particles (VLPs) that elicit host protective neutralizing antibody (Frazer, 2009). The PV VLPs showed high immunogenic proprieties and have been experimentally used in several studies on different animal models. In this context, the antibody titers after immunization with PV VLPs are 80- to 100-fold higher than those measured following natural infection. Additionally, the immunization of animals with recombinant L1

human PV VLPs is able to generate neutralizing antibodies against the wild-type virus and protected vaccinated animals against experimental infection with the homologous papillomavirus (Nayereh and Khadem, 2012). For this reason, the majority of prophylactic vaccines have been developed targeting the major capsid protein L1 of the viral particle. These vaccines induce a polyspecific antibody response which recognizes a range of conformational determinants on the viral capsid (Frazer, 2009).

Conversely, the aim of therapeutic vaccines is the development of an immune responses able to eliminate the cells already infected with the virus. These vaccines are designed to prime the antigen-specific T-cell mediated mechanisms for controlling and eliminating the viral infections. In this context, the E6 and E7 proteins are the principal targets, since they are expressed early in viral infection and are essential for transformation of the infected cells (Nayereh and Khadem, 2012).

Various types of therapeutic vaccines against PV-associated lesions have been developed, including live vector-, peptide- and nucleic acid-based vaccines.

Live vector-based vaccines are categorized as bacterial or viral vectors depending on their vector platform. These therapeutic vaccines are highly immunogenic and can induce a strong cellular and humoral immune response. Additionally, they can also stimulate antigen presentation through MHC class I and II by the delivery of the E6 and E7 antigens to APCs. Nevertheless, live vector-based vaccines represents a potential risk in immunocompromised individuals (Yang *et al.*, 2016). Protein-based vaccines provides the direct administration of peptides derived from PV antigens. PV antigenic proteins are taken up by dendritic cells and presented in association with the MHC class I and/or class II pathways on human leukocyte antigen (HLA) molecules, to mount an immune response against the pathogen. The problem of these vaccines is due to the identification of specific immunogenic epitopes of PV antigens that is necessary before

vaccine development, although is difficult to produce a peptide-based vaccine that is effective in patients with different HLA haplotypes. Additionally, the protein-based vaccines also suffer from low immunogenicity and adjuvant and fusion protein strategies are often used to enhance vaccine potency (Hung *et al.*, 2008). Nuclei acid-based vaccines are categorized as DNA- or RNA-based vaccines. DNA vaccines have emerged as an effective strategy for antigen-specific immunotherapy, since the DNA is stable and can be used to sustain the expression of antigen in cells for longer periods of time than RNA or protein vaccines. Furthermore, unlike live-vector vaccines, DNA vaccines do not elicit neutralizing antibody production, permitting repeated vaccinations. Similarly, RNA-based vaccines do not lead to the generation of neutralizing antibodies, since they do not form viral particles. RNA vaccines can be delivered from several RNA viruses. RNA replicon are capable of self-replication, leading thus sustained level of antigen expression and increased immunogenicity (Yang et l., 2016).

1.4 Animal Papillomavirus

In animals, 137 non-human PV types were recovered from 54 different host species, including mammals, birds and reptile species, and the complete genomes of these PVs are reported on GenBank. Animal PVs are distributed over 32 different genera, leaving only the genus *Gamma*, *Mu* and *Nu* papillomavirus that contain exclusively human PV types.

Cynomolgus macaques, domestic cows, and dogs, are well-studied species and are of particular interest for comparative study in human. A high number of different PV types have been discovered in these vertebrate species. However, recent advances in cloning and sequencing methods have enabled to exponential increase the number of completely characterized animal PV genomes also in the other species (Rector and Van Ranst, 2013).

1.4.1 Primate papillomaviruses

In primate species, several PV types have been isolated from cutaneous and mucosal lesions. A high phylogenetic relation between human and primate papillomavirus has been established, leading to classify mostly of primate PV into the *alpha* genus. The *alpha* genus represents a significant medical genus containing the oncogenic genital PV types associated with all cases of human cervical, anal, penile, head and neck cancer (Bergin *et al.*, 2013). Cynomolgus macaque (*Macaca fascicularis*), representing the most studied primate species, nine PV types, (MfPV3-MfPV11), have been isolated from cervical intra-epithelial neoplasia and classified into the *alpha* genus (Chen *et al.*, 2009). Cervical neoplasia occurs relatively frequently in female macaques and the possible viral involvement was firstly reported by Ostrow and colleagues in 1991. This

PV has been named MmPV1 and has been classified in the alpha genus. Furthermore, PV infection was also revealed in a cervical samples (CIN1) of a Hamadryas baboon (Papio hamadryas Anubis). This PV, included in the alpha genus, was named PhPV1 (Bergin *et al.*, 2013). More recently, Wood and colleagues (2004) showed the PV presence in lesions sharing histopathologic similarities with those found in women. The same group reported a strong phylogenetic relation between MfPV3 and Human PVs (HPV) and shared the experimental transmission of this virus, suggesting the female macaques as highly relevant preclinical model for the study of HPV biology (Wood *et al.*, 2007).

Evidence of primate PV infections were reported also in oral mucosa. In particular, in oral epithelial hyperplasia of a pygmy chimpanzees (*Pan paniscus*) was identified the PpPv1 and classified into the alpha genus (Van Ranst *et al.*, 1991). Interestingly, this PV type resulted evolutionary related to HPV 13, which is associated with oral focal epithelial hyperplasia in humans. Also, in the Colobus monkey (Colobus guereza), two different types of PV were isolated from papillomas and named CgPV1 and CgPV2 (O'Banion *et al.*, 1987; Kloster *et al.*, 1988). CgPV1, classified in the alpha genus, was isolated from a penile papilloma, while CgPV2, belonging to the beta genus, was detected in a cutaneous papillomas. Primate PV belonging to the beta genus, include also two additionally PV types, named MfPV1 and MfPV2, isolated in cutaneous papilloma of a Cynomolgus macaque (*Macaca fascicularis*) (Joh *et al.*, 2009; Wood *et al.*, 2011).

1.4.2 Bovine Papillomaviruses

In cattle, fourteen bovine Papillomaviruses (BPVs) have been characterized and classified into three *genera*:

- ✓ *Delta genus*, including BPV1, BPV2, BPV13 and BPV14

- ✓ *Epsilon genus*, including BPV5 and BPV8
- ✓ *Xi genus*, including BPV3, BPV4, BPV6, BPV9, BPV10, BPV11 and BPV12

BPV1 and BPV2 of the *Delta* genus are associated with cutaneous fibropapilloma in cattle, while BPV13 and BPV14 has been recently identified in a cutaneous papilloma and urinary bladder tumor, respectively (Chen *et al.*, 1982; Lunardi *et al.*, 2013; Roperto *et al.*, 2016).

Macroscopically, fibropapilloma appears as an exophitic papillary mass protruding from the skin (Fig 1.6 a). Histologically, fibropapillomas are characterized by a proliferation of mesenchymal cells, covered by hyperplastic epithelium that may contain koilocytosis and large cells often exhibiting blue-gray cytoplasm (Fig 1.6 b).

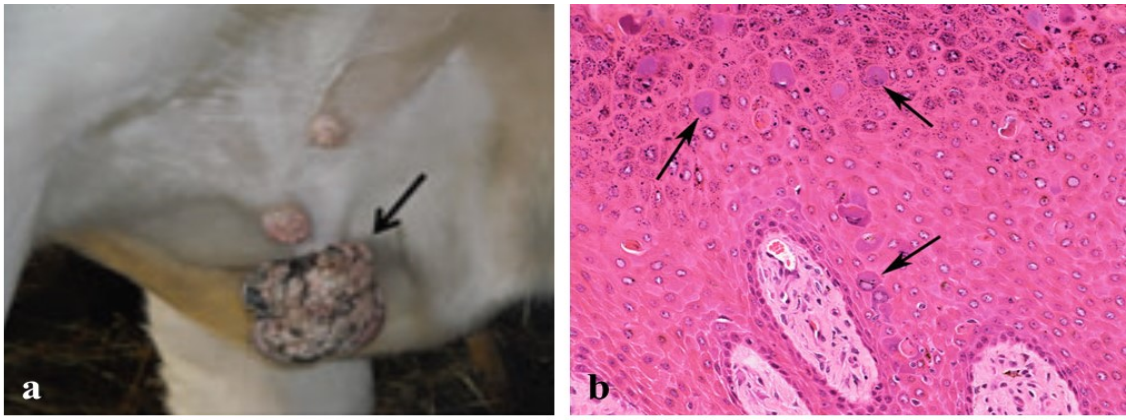

Fig 1.6 Bovine cutaneous fibropapilloma. **(a)** Macroscopic appearance of a cutaneous fibropapilloma (Bocaneti *et al.*, 2016). **(b)** Histological features of a cutaneous fibropapilloma characterized by a hyperplastic epithelium showing numerous large cells with blue-gray cytoplasm (H&E) (Munday, 2014a).

Although BPV1 and BPV2 are able to infect both mesenchymal and epithelial cells population, the productive viral replication is limited to the epithelium (Munday, 2014a). A predominance expression of the E5 protein and a lesser role of both E6 and E7 proteins occurs during a productive infection by BPV1 and BPV2, indicating a key role of this protein in tumor development (Silvestre *et al.*, 2009; Munday, 2014a).

Although sequence similarity between BPV1 and BPV2 of the *Delta* genus, BPV2 showed different behavioral characteristics and is able to replicate in a wide range of tissues, as the transitional epithelium of the bladder (Roperto *et al.*, 2008; Roperto *et al.*, 2013). In particular, BPV2, as well as BPV14, seem involved in urinary bladder tumors development. Lesions are both of epithelial and mesenchymal origin with different histological variants identified. Roperto and colleagues (2010) established a histological classification system for bovine bladder tumors based on the World Health Organization (WHO) scheme. This classification identify four distinct growth patterns of bovine urothelial tumors and tumor-like lesions, classify as flat, exophytic or papillary, endophytic and invasive lesions. BPV2 has been detected in all these lesions, suggesting thus an association between PV infection and bladder cancer, independently of the histotypes (Bocaneti *et al.*, 2016). Nevertheless, BPV-2 infects the urinary bladder mucosa inducing an abortive and latent infection with no production of virions. The exposure to immunosuppressants, mutagenic and carcinogenic principles from bracken triggers viral gene expression, leading to cell transformation (Borzacchiello 2007). Despite this evidence, the rarity of bovine bladder neoplasia in geographical areas without bracken fern leads very difficult determine the contribution of each factor in the process of carcinogenis.

BPV5 and BPV8 of the *epsilon* genus show different behavioral characteristics. In particular, BPV5 is associated with fibropapillomas development in teats, udder and face, while BPV8 is responsible of cutaneous papillomas. Papillomas are benign exophitic proliferations of the epithelium, usually showing a spontaneously regression in cows or an evolution in extensive papillomatosis in debilitated animals, resulting in multiple lesions (Fig 1.7 a) (Bocaneti *et al.*, 2015). Histologically, lesions appear as a proliferation of tumor cells producing finger-like or warty projections with a consequent

increasing in layers number (Fig 1.7 b). As the fibropapillomas, papillomas are caused by all BPV type, with the exception of BPV-4. During papilloma formation, the expression of BPV genes is strictly regulated, leading the initial expression of early genes in basal and suprabasal layers. Interestingly, the basal and superficial localization of BPV-E5 protein in viral infection indicates that the E5 may have a key role in both early and late stage, as well as in tumors development (Corteggio *et al.*, 2013).

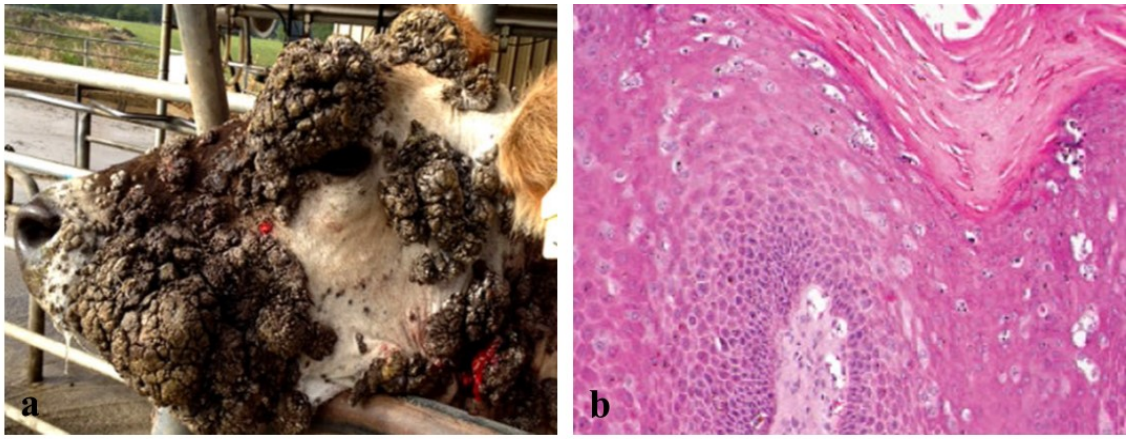

Fig 1.7 Bovine cutaneous papilloma. **(a)** Presence of extensive papillomatosis on the head of a cow (Munday, 2014a). **(b)** Histological appearance of cutaneous papilloma characterized by epidermal hyperplasia and compact orthokeratotic hyperkeratosis (H&E) (Goldschmidt and Goldschmidt, 2017)

In the *xi* genus, the majority of BPVs are responsible of cutaneous papillomas, except BPV4. This PV type is associated to the papillomas development in upper alimentary tract, particularly in oral cavity, esophagus and rumen (Fig 1.8 a) (Munday, 2014a). Histologically, lesions show the classical features of benign papillomas with multiple papillary fronds covered by the multiple-layers of keratinised epithelium (Fig 1.8 b). Epithelial acanthosis and parakeratosis are also observed and the active viral replication results in inclusion bodies visible in the nuclei of infected cells (Borzacchiello and Roperto, 2008). Rates of disease are dependent on bracken fern exposure, whose immunosuppressive properties are thought to predispose to papilloma development

(Munday, 2014a). Interestingly, infection by BPV-4 may also evolve in squamous cell carcinoma (SCC) of the upper alimentary tract, with a similar distribution of the papillomas (Munday, 2014a). Nevertheless, as reported by Campo (1985) and by Anderson and colleagues (1997), no viral DNA or PV protein are detected in in this tumor.

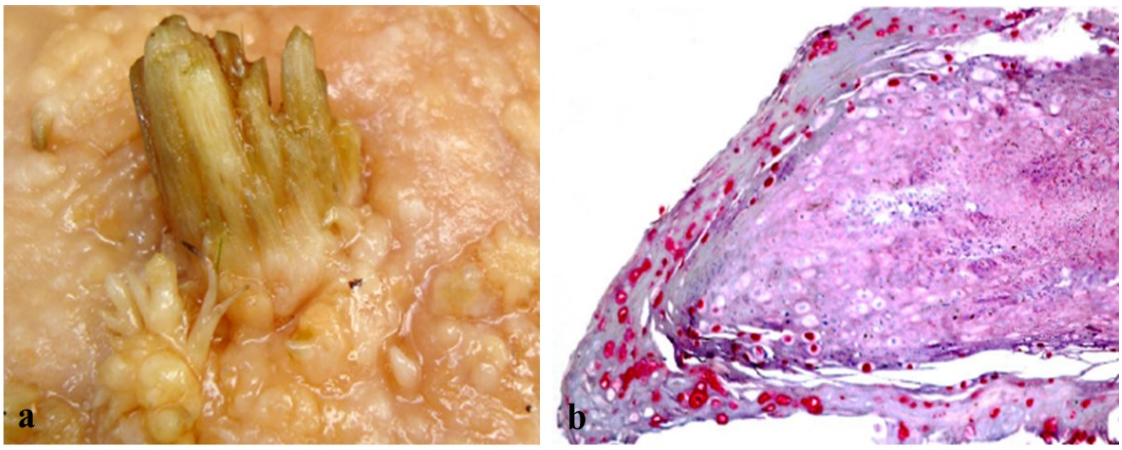

Fig 1.8 Bovine oropharyngeal papillomas. **(a)** Macroscopic appearance of lesion with oropharyngeal localization in a cow (Munday, 2014a). **(b)** Histological features of lesion and detection of PV L1 protein by immunohistochemistry (V-Red detection chromogen with hematoxylin counterstain) (Munday, 2014a).

1.4.3 Bovine Papillomaviruses in equids

Although PVs are considered species specific, BPV1, BPV2 and rarely BPV13, can jump the species barrier and are associated with the pathogenesis of equine sarcoids (Yuan *et al.*, 2007; Gaynor *et al.*, 2015). The association between sarcoids and BPVs was first observed in horses intradermal inoculated with a cell-free extract from cattle warts, resulting in the growth of sarcoid-like lesions (Olson and Cook, 1951). Subsequently, several studies reported a high detection of BPV1 and BPV2 DNA in examined sarcoids tumors and showed the detection of viral oncogenes and capsid gene transcripts of BPV, providing further evidence for the direct viral involvement in the pathogenesis of sarcoids (Taylor and Haldorson, 2013).

Equine sarcoids is the most commonly skin tumor reported in horses, donkeys and mules (Lunardi *et al.*, 2013). The term sarcoid is referred to a spectrum of locally aggressive nonmetastasizing skin tumors of both epithelial and mesenchymal components (Fig 1.9 a) (Gaynor *et al.*, 2015). Sarcoids can occur as single or multiple lesions in different forms, ranging from small wart-like lesions to large ulcerated fibrous growths (Chambers *et al.*, 2003). Histologically, tumor is characterized by epidermal hyperplasia and hyperkeratosis with epithelial extensions projecting into the underlying dermis (Fig 1.9 b). Additionally, proliferating dermal fibroblasts forming whorls or interlacing bundles are observed in tumor.

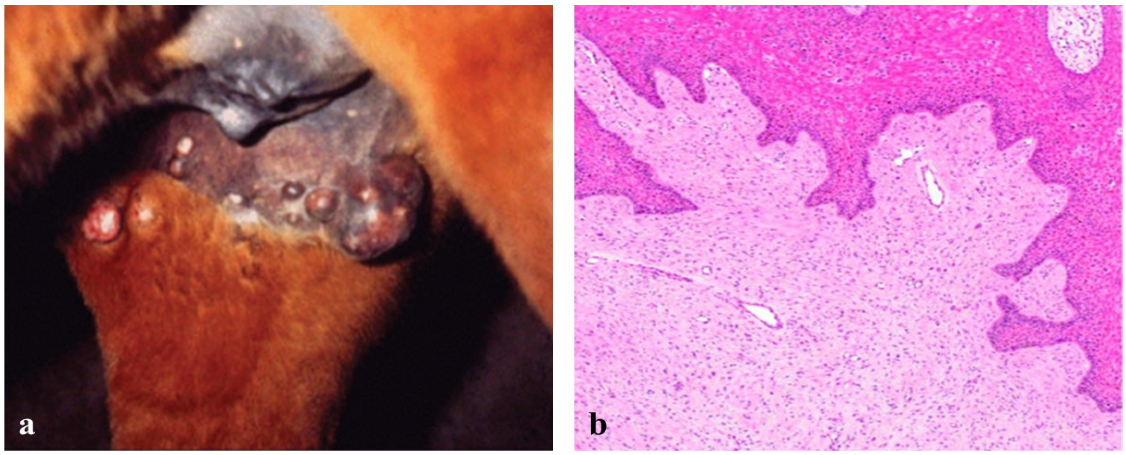

Fig 1.9 Equine sarcoids. **(a)** Macroscopical appearance of a fibroblastic sarcoids in the hind legs of a horse (Chambers *et al.*, 2003). **(b)** Histological features of equine sarcoids showing hyperplastic epidermis overlying a nodular accumulation of fibroblasts (H&E) (Gaynor *et al.*, 2015).

The molecular events leading to equine sarcoids are poorly understood. It is not known how horses become infected by BPV, although insects have been proposed as vectors of BPV in disease transmission (Taylor and Haldorson, 2013). Additionally, it has been proposed that the transmission of infection occurs between equids and not between cattle and horses, due to the absence of BPV-1 in bovine papillomas (Nasir *et al.*, 2007). These hypothesis appeared further validated by the identification of several difference

in sequence analysis of BPV DNA extracted from sarcoids compared to BPV1 DNA extracted from bovine bladder cancer (Chambers *et al.*, 2003). Numerous variants of the BPV1 E5 ORF have been identified in equine sarcoids, suggesting thus an influence in cellular location and oncoproteins function, leading to a more efficient cell transformation in equine species (Nasir *et al.*, 2007). Furthermore, it seems that there is a breed predisposition in Quarter Horses, Arabians and Appaloosas and an increased risk with the presence of the MHC alleles A3 and W13 (Angelos *et al.* 1988; Taylor and Haldorson, 2013). Although it is widely accepted that BPV infection is necessary for sarcoids development, infection alone could be not sufficient for tumor production (Bogaert *et al.*, 2008).

1.4.4 Equine Papillomaviruses

In horses, PV associated skin disorders are common and occur in different forms. To date, seven different PVs have been isolated from different mucosal and cutaneous lesions and classified as follows:

- ✓ *Zeta* genus, including the equus caballus papillomavirus 1 (EcPV1)
- ✓ *Dyoiota* genus, including EcPV2, EcPV4 and EcPV5
- ✓ *Dyorho* genus, including EcPV3, EcPV6 and EcPV7

EcPV1 was isolated in a cutaneous papilloma, the most common tumor in horses between 1 and 3 years of age, particularly affecting nose and lips (Ghim *et al.*, 2004; Postey *et al.*, 2007). Evidence for a causal link between PV and disease is limited, although PV virions have been observed in nuclei of cells of the stratum granulosum, corneum, and spinosum by electron microscopy, and PV antigen has been identified with immunohistochemical techniques (Postey *et al.*, 2007).

EcPV2 of the *Dyoiota* genus was firstly isolated in genital papillomas. Recently, Lange and colleagues (2012) reported the presence of this PV in both *in situ* and invasive SCC of the penis. Typical histological findings associated to PV-infection (koilocytes) were observed in analyzed cases and the EcPV2 DNA was detected by probe hybridization in superficial cells of the epidermis, suggesting a direct PV-involvement in the development of lesions (Lange *et al.*, 2012).

EcPV4 and EcPV5 of the *Dyoiota* genus were isolated from a vulvar and aural plaque, respectively (Lange *et al.*, 2013). In particular, aural plaque can occur in horse of all age as well-demarcated raised, depigmented, hyperkeratotic plaques on the inner surface of the ear pinnae. A spontaneously regression of this lesions is extremely rare and, conversely, secondary infection can frequently occur (Postey *et al.*, 2007).

Similar to EcPV5, EcPV3 and EcPV6 of the *Dyorho* genus were isolated from aural plaque (Lange *et al.*, 2011; Lange *et al.*, 2013). Conversely, EcPV7 was identified in a penile mass (Lange *et al.*, 2013)

1.4.5 Canine Papillomaviruses

In dog, twenty PV types have been fully sequences and classified into three different genera:

- ✓ *Lambda* genus, including CPV-1 and CPV-6
- ✓ *Tau* genus, including CPV-2, CPV-7, CPV-13, CPV-17 and CPV-19
- ✓ *Chi* genus, including CPV-3, CPV-4, CPV-5, CPV-8, CPV-9, CPV-10, CPV-11, CPV-12, CPV-14, CPV-15, CPV-16, CPV-18 and CPV-20

CPV1 of the *Lambda* genus, originally denominated canine oral papillomavirus (COPV), induces papillomas at the mucous membrane of the oral cavity and at the haired skin of dogs (Bernard *et al.*, 2010; Lange and Favrot, 2011). Canine oral

papillomatosis caused by CPV1 is more frequent in dogs of approximately one year of age and typically causes cauliflower-like exophytic warts. Lesions are normally localized in oral mucosa, lips and mucocutaneous junctions, but are not restricted to those sites. Although CPV1 is typically related to benign and spontaneously regressing papillomas, this viral type has been occasionally reported in non-regressing lesions, particularly in oral and perioral squamous cell carcinomas (Porcellato *et al.*, 2014).

Furthermore, CPV-1, together with CPV-6, are both associated with endophytic warts (Fig 1.10 a). Lesions results in nodules with a central pore filled with keratin and are characterized by a growth downward into the skin. Histologically, endophytic papillary projections of the epidermis extending into the dermis are typical and cytophatic effects, as koilocytes and basophilic or eosinophilic inclusions, are usually present (Fig 1.10 b) (Lange and Favrot, 2011).

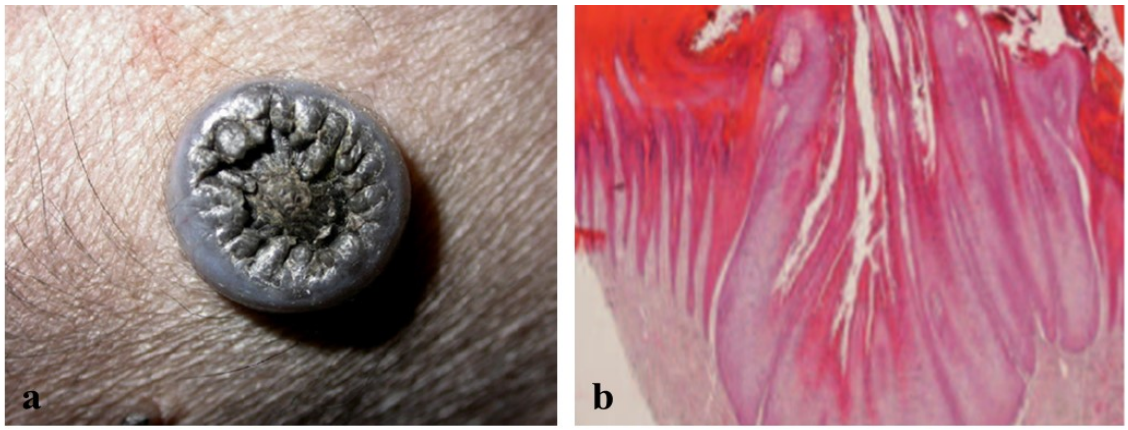

Fig 1.10 Canine endophytic warts. **(a)** Macroscopic appearance of lesion localized in the abdomen of a dog (Lange and Favrot, 2011). **(b)** Histological features of entophytic wart characterized by papillary projections of the epidermis into the dermis (H&E) (Lange *et al.*, 2009).

CPV2 and CPV-7 of the *Tau* genus has already been isolated from cutaneous exophytic papillomas. These lesions may develop in dogs of any age and are present as single or multiple skin lesions mainly found on the head, eyelids, and feet. Lesions are white,

pink, or pigmented papillated masses that may be sessile or pedunculated. Microscopically, cutaneous exophytic papilloma consists of marked epithelial proliferations on numerous thin fibrovascular stalks. Cutaneous exophytic papillomas may persist for several months or regress spontaneously over a period of weeks to months (Nagata and Rosenkrantz, 2013).

Additionally, CPV-2 has been reported in endophytic papillomas, as well as in an invasive squamous cell carcinoma, whereas CPV-7 has been reported in a bowenoid in situ carcinoma.

Conversely, CPV-13, CPV-17 and CPV-19 are associated with mucosal lesions. In particular, CPV-13 and CPV-19 has been isolated from oral papillomatosis and CPV-17 from oral SCC (Munday *et al.*, 2016).

CPVs of the *Chi* genus are associated with cutaneous pigmented plaques, commonly found in pugs and miniature schnauzers during young adulthood (Lange *et al.*, 2013). Pigmented plaques may be an inherited autosomal dominant trait and several studies reported an increased incidence of this papillomatous lesion in immunocompromised animals (Nagata and Rosenkrantz, 2013). Lesions are common in the limbs, axillae, or abdomen and consist in multiple dark, plaquelike hyperkeratotic lesions (Fig 1.11 a) (Lange and Favrot, 2011). Histologically, plaques are characterized by demarcated, irregularly acanthosis with marked hyperkeratosis and hyperpigmentation (Fig 1.11 b) (Nagata *et al.*, 2013. Compendium of dermatology). Lesions develop progressively over time and generally do not regress.

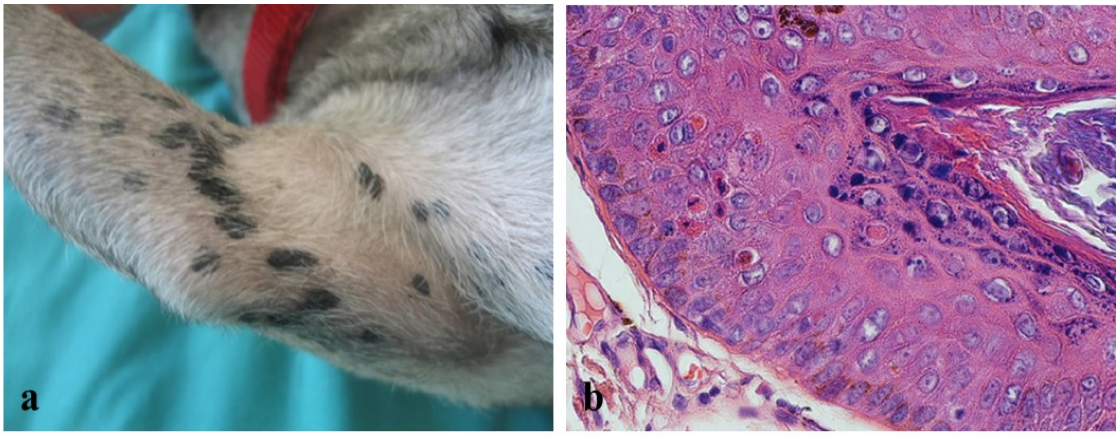

Fig 1.11 Canine pigmented plaques. **(a)** Macroscopic appearance of pigmented plaques localized in the axillae of a dog (Lange and Favrot, 2011). **(b)** Histological feature of pigmented plaques characterized by the presence of koilocytes and viral inclusion (H&E) (Lange and Favrot, 2011).

1.4.6 Feline Papillomaviruses

In domestic cat, four PV types have been fully classified, including Felis catus PV-1 (FcaPV-1) from a cutaneous viral plaque, FcaPV-2 and FcaPV-3 from cutaneous bowenoid in situ carcinomas, and FcaPV-4 from a sample of gingivitis (Munday *et al.*, 2015).

FcaPV-1, a *lambda*-PV closely related to canine PV-1, was firstly identified in a cutaneous plaque (Tachezy *et al.*, 2002). Nevertheless, recent investigation on a higher number of cutaneous hyperkeratotic lesions reported a high prevalence of FcaPV-2, leading to consider FcaPV-2 the most common cause of these lesions in domestic cats (Munday and Peters-Kennedy, 2010). Nevertheless, the epidemiology and pathogenesis of viral plaques in cats remains unclear. Although there is some evidence that immunosuppression may predispose cats to plaque formation, plaques also develop in cats without any detectible immunosuppressive disease (Munday and Peters-Kennedy, 2010). Cutaneous viral plaque can be pigmented or non-pigmented, usually covered with a keratin crust, and typically present as multiple, non-painful, ovoid, <8 mm

diameter lesions (Fig 1.12 a). Histologically, lesions do not exhibit significant cell dysplasia, while prominent viral cytopathology is visible in the mildly thickened epidermis (Fig 1.12 b) (Munday, 2014b).

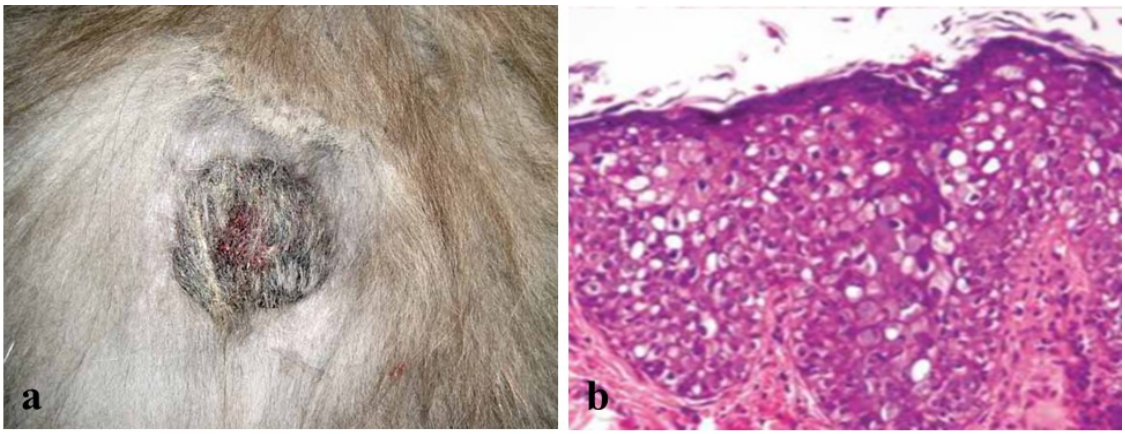

Fig 1.12 Feline cutaneous viral plaques. **(a)** Macroscopic appearance of viral plaque localized on the trunk of a cat (Nagata and Rosenkrantz, 2013). **(b)** Histological features of viral plaque, showing a moderate epidermal hyperplasia (H&E) (Munday *et al.*, 2008).

FcaPV-2 and FfcaPV-3 have been identified in bowenoid in situ carcinomas (BISCs) and classified in the *dyotheta* and *tau* genus, respectively (Bernard *et al.*, 2010; Munday *et al.*, 2013). BISCs usually present as multiple crusting or ulcerated plaques (Fig 1.13 a), histologically characterized by epidermal thickening and dysplasia, extending into hair follicles and confined by the basement membrane (Fig 1.13 b). Dysplastic cells exhibit elongated nuclei, usually orientated in one direction and with evident viral cytopathology. BISCs are rare lesions that can develop as progressions from cutaneous viral plaques, although the mechanism is still unknown. However, a BISC can develop de novo, without the presence of precursor lesions, and a proportion of this tumors can progress to squamous cell carcinoma (Munday, 2014b). The BISC progression in squamous cell carcinoma (SCC), led the hypothesis of a viral involvement in this malignant tumor. SCC lesions containing FcaPV-2 have been reported in several studies, whereas a direct viral involvement in the pathogenesis of this tumors has not

been identified, suggesting the recruitment of additional cofactors in the development of neoplasia (Munday, 2014b).

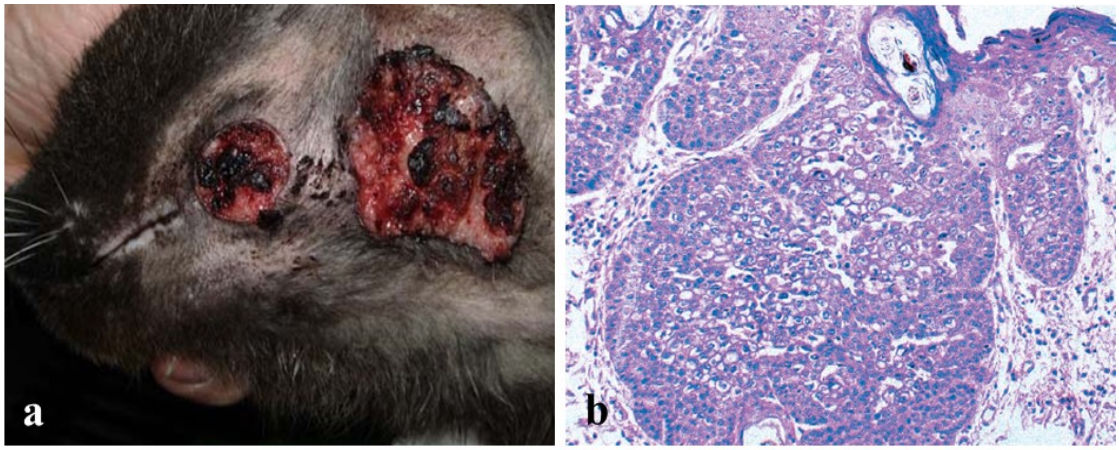

Fig 1.13 Feline bowenoid in situ carcinomas. **(a)** Macroscopic appearance of lesions localized on the haired skin of the mandible of a cat (Nagata and Rosenkrantz, 2013). **(b)** Histological features of BISC characterized by irregular hyperplasia of the epidermis (H&E) (Favrot *et al.*, 2009).

FcaPV-4, classified in the *tau* genus, was firstly identified in a domestic cat with severe gingivitis (Dunowska *et al.*, 2014). Nevertheless, a recent investigation suggested that FcaPV-4 is not the only feline PV type involved in mucosal lesions. In fact, the detection of FcaPV-1 in two oral papillomas of domestic cats leads to hypotize this PV as the most likely cause of the feline oral papillomas (Munday *et al.*, 2015). Although FcaPV-1 was initially detected in a feline cutaneous viral plaque, its detection in oral lesions seems to confirm the relationship with the *Lambda* genus, reported to cause oral papillomas in exotic cats.

1.4.7 Caprine Papillomaviruses

Recently, one PV, named *Capra hircus* PV-type 1 (ChPV-1), has been completely sequenced from healthy goat skin and classified in the *Phi* genus (Van Doorslaer *et al.*, 2006). Although a report of goat papillomatosis has been reported in 1954, no sequence

data of this putative goat PV have been determined and its complete genomic DNA has never been amplified (Moulton, 1954). More recently, PV DNA sequences have been detected in papillomatous lesions from the mammary skin of goats (Manni *et al.*, 1998). Additionally, the presence of PV DNA have been detected in multiple ocular and cutaneous neoplastic lesions spontaneously occurring in two adult Maltese twin goats (Simeone *et al.*, 2008). This study represents the first report of PV-associated multiple cutaneous and ocular tumors in goat. PV infection has been proposed as cofactors of other established risk factors in skin carcinogenesis, such as sunlight exposure and immunosuppression. Similarly to the ovine species, no additional studies has yet been performed in order to elucidate the role of PV infection also in caprine species.

1.4.8 Ovine Papillomaviruses

In sheep, three PV types have been fully sequenced and classified into two different genera. In particular, *Ovis aries* Papillomavirus 1 (OaPV1) and 2 (OaPV2), have been historically described in Australia in cutaneous fibropapilloma and classified into the *Delta* genus, while OaPV3 (GenBank accession n# FJ796965) has been recently identified in cutaneous squamous cell carcinoma of Sardinian sheep and placed in the genus *Dyokappa*. The genomic characterization of OaPV3 showed important differences if compared to previously reported ovine PVs, including the presence of gene codified proteins potentially involved in malignant tumors development (Alberti *et al.*, 2010). Nevertheless, no additional studies have been performed in order to investigate the viral activity in ovine tumor.

Cutaneous squamous cell carcinoma (SCC) is a malignant neoplasm of epidermal cells in which the cells show differentiation to keratinocytes (Goldschmidt and Goldschmidt, 2017). SCC is the most common form of skin cancer in sheep, reported in Australia,

South Africa, Italy, France, Spain, Saudi Arabia and Brazil (Alberti *et al.*, 2010). Tumors development can be preceded by actinic keratosis, a chronic reactive proliferation of keratinocytes with a different degree of dysplasia and inflammatory response. Macroscopically, SCC typically appears as cutaneous horn, crateriform, papillary, or fungiform masses, frequently observed in pinnae, eye, and udder and less commonly in muzzle, lower lip, and vulva (Fig 1.17 a) (Ahmed and Hassanein, 2012). Tumors are usually slow growing and most neoplasms, although invasive, do not show metastatic spread to regional lymph nodes.

Two variants of neoplasia can be observed, based on the histological grade of tumor differentiation. Well-differentiated tumors are characterized by islands or trabeculae of squamous epithelial cells showing an orderly differentiation from non keratinized to keratinized cells. A central accumulations of compact laminated keratin, or keratin pearls, are present in variable size and numbers (Fig 1.14 b). Mitotic activity is moderate and a stromal infiltration of inflammatory cells is generally present. Poorly differentiated SCC show a similar architecture characterized by the presence of small cords or nests of cells showing prominent and hyperchromatic nuclei (Fig 1.14 c). The mitotic activity is high and keratin pearls are not observed.

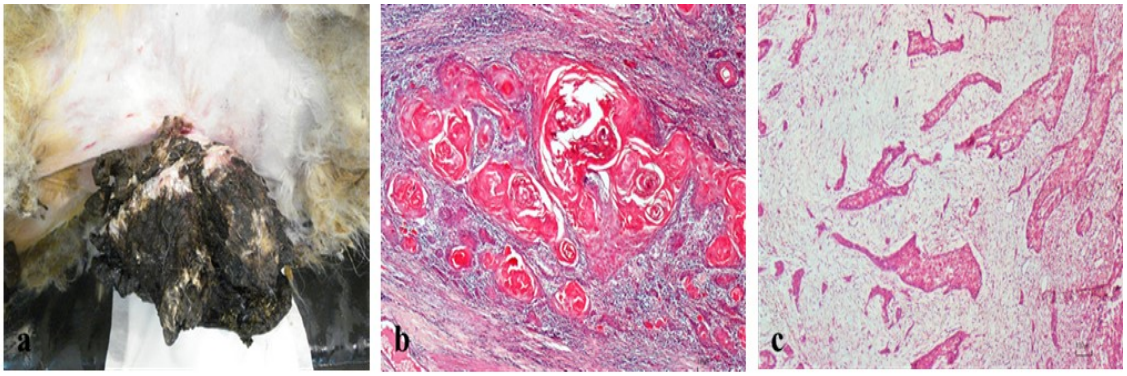

Fig 1.14 Cutaneous squamous cell carcinoma in sheep. **(a)** Macroscopic appearance of cutaneous SCC in the lower flank of sheep (Ahmed and Hassanein, 2012). **(b)** Histological features of well differentiated SCC (H&E). **(c)** Histological features of poorly differentiated SCC (H&E).

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Aim of the thesis

Papillomaviruses (PVs) are associated with benign and malignant tumor of the skin and mucous membranes in humans and the viral infection is considered a driving force of neoplastic transformation. In veterinary medicine, the role of papillomaviruses as the causative agents of malignant transformation is well known in bovine, equine, and canine species, whilst in sheep has been marginally investigated. Nevertheless, the recent identification of a new ovine PV types in cutaneous squamous cell carcinoma opens new research perspectives.

Squamous cell carcinoma is the most common form of skin cancer in sheep and the etiology has been linked to ultraviolet radiation and poor skin pigmentation. The study of PVs in cutaneous malignant lesions, aimed to clarify the role of PV infection in sheep, could provide new insight into the etiology of ovine skin cancer. Furthermore, the identification of molecular pathways involved in skin cancer development can elucidate the viral-host interaction, providing additional evidence of the PV role in tumors onset.

Based on these considerations, the aim of the present thesis was to:

- ✓ clarify the etiology of cutaneous squamous cell carcinoma in sheep, investigating the presence, the localization and the transcriptional activity of PVs by an integrated histological and biomolecular approach
- ✓ identify the pathways involved in viral neoplastic transformation and discovery new putative biomarkers related to the viral infection in cutaneous squamous cell carcinoma by proteomic analysis.

Chapter II

Ovis aries papillomavirus 3 in ovine cutaneous squamous cell carcinoma

2.1 Introduction

Papillomaviruses (PVs; family *Papillomaviridae*) are highly species-specific viruses able to infect mammals, birds and reptile species (Zheng and Baker, 2006). PVs are small and non-enveloped viruses with a highly conserved circular double-stranded DNA genome of approximately eight-kilobases in length (de Villiers *et al.*, 2004; Rector and Van Ranst, 2013). The majority of PVs infect the basal layer of the stratified epithelium and replicate exclusively in epithelial cells, with a life cycle tightly regulated by cellular differentiation (Doorbar, 2005; Mistry *et al.*, 2008; Munday, 2014; Bravo and Felez-Sanchez, 2015). Moreover, a small number of PVs infect and transform fibroblast cells (Gaynor *et al.*, 2016). The epithelial infection usually begins from cutaneous or mucosal surface lesions and results in benign proliferative lesions or, rarely, malignant cancers (Zheng and Baker, 2006; Doorbar *et al.*, 2012; Doorbar, 2016).

In animals, 112 PV types distributed over 32 genera have been characterized based on their well-conserved L1 nucleotide sequence (de Villiers *et al.*, 2004; Bernard *et al.*, 2010; Rector and Van Ranst, 2013). In sheep (*Ovis aries*), two PVs, named Ovis aries papillomavirus 1 (OaPV1) and 2 (OaPV2), have been described in Australia and classified into the *Delta* genus. These viruses are associated with cutaneous fibropapillomas, although their viral activity in benign lesions has been poorly investigated in sheep. In cows, bovine PVs of the *Delta* genus are responsible for cutaneous fibropapillomas, which spontaneously regress in immunocompetent animals but can potentially progress to squamous cell carcinoma (Borzacchiello and Roperto, 2008; Nasir and Campo, 2008; Bocaneti *et al.*, 2016).

Recently, in Sardinia (Italy), a novel epidermotropic ovine PV, Ovis aries papillomavirus 3 (OaPV3), has been described in two ovine SCCs (Alberti *et al.*, 2010).

The genomic characterization of OaPV3 (GenBank accession n# FJ796965) showed a low degree of similarity (less than 60%) with other PVs and was placed in the genus *Dyokappa*. Unlike the *Delta* papillomavirus, the OaPV3 genome lacks the E5 gene and retains the conserved retinoblastoma tumor suppressor binding sequence motif in E7, suggesting that this virus could play a key role in malignant cancer development (Alberti *et al.*, 2010; DiMaio and Petti, 2013).

SCC, a malignant tumor of epithelial origin with differentiation to keratinocytes, is widely described in animals and is the most common form of skin cancer in sheep (Mendez *et al.*, 1997; Alberti *et al.*, 2010). The cause of this neoplasm appears to be multifactorial, although prolonged exposure to ultraviolet radiation and poor skin pigmentation are considered primary risk factors for tumor development (Markey, 1995; Marks, 1996; Mendez *et al.*, 1997; Ahmed and Hassanein, 2012). However, oncogenic viruses, such as PVs, seem to be associated with the cutaneous SCC development in human and animal species, suggesting a possible correlation between the infection and carcinoma (Munday and Kiupel, 2010; Aldabagh *et al.*, 2013; Wang *et al.*, 2014). The aim of this study was to investigate the presence of OaPV1, OaPV2 and OaPV3 in SCC samples of Sardinian sheep by histological and biomolecular analysis.

2.2 Material and methods

2.2.1 Origin of the samples

Forty cutaneous SCC and 40 matched non-SCC samples from 70 sheep of the Sardinian breed, obtained from udder (SCC= 21; non-SCC=21), eyelid (SCC=7; non-SCC=7), pinnae (SCC=5; non-SCC=5), planum nasale (SCC=4; non-SCC=4) and trunk (SCC=3; non-SCC=3), were fixed in 10% neutral buffered formalin and then paraffin embedded (FFPE). Tissue sections (3µm thick) from both SCC and non-SCC samples were stained with hematoxylin and eosin (HE) for histopathological evaluation and 10 serial sections from each FFPE sample were collected in 1.5 ml tube and used for DNA and RNA isolation. Additionally, FFPE SCC samples were used for *in situ* hybridization (ISH).

2.2.2 Histopathology

Tumors were classified according to World Health Organization criteria for tumors of the Skin and Soft Tissue and graded according to a semi-quantitative scheme, originally developed for oral SCC in dogs, with minor modifications (Anneroth *et al.*, 1987; Misdorp, 1999). Degree of keratinization, nuclear pleomorphism, number of mitosis, pattern of invasion and lymphoplasmacytic infiltration, were each assigned a score ranging from 1 to 4 points. Histopathological features were evaluated in 10 high-power fields and the sum of each parameter sub-classified SCCs as follows: grade I or well differentiated (WDC), 1-7 points; grade II or moderately differentiated (MDC), 8-14 points; grade III or poorly differentiated (PDC), 15-20 points. The stained slides were reviewed independently by three pathologists (G.P.B, E.A. and S.P.) and a consensus score was obtained for each case on a multiheaded microscope. Digital computer images were recorded with a Nikon Ds-fi1 camera.

2.2.3 Detection of ovine PVs

DNA from SCC and non-SCC FFPE samples was extracted using the AllPrep DNA/RNA FFPE kit (Qiagen, Milano, IT), following the manufacturer's instructions.

The quality of the DNA samples was checked by PCR amplification of the ovine glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene (Budhia *et al.*, 2006). The amplification program consisted in an initial denaturation step at 94°C for 5 minutes, followed by 30 thermal cycles at 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec, with a final elongation step at 72°C for 5 min.

Subsequently, in order to evaluate the presence of ovine PVs (OaPV1, OaPV2, OaPV3) in both SCCs and non-SCC samples, three distinct PCR assays for detection of the L1 gene, were performed in a total volume of 50 µl containing 1.5 mM $MgCl_2$, 0.2 µM of each oligonucleotide primer, 0.2 mM of each dNTP, 0.62 U of Taq DNA polymerase (Qiagen, Milano, IT) and 2.5 µl of DNA. Each reaction was carried out using primers set listed in Table 2.1.

Reaction conditions for OaPV1 and OaPV2 included initial denaturation at 95°C for 5 min, followed by 40 cycles at 95°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 30 s, with a final elongation step at 72°C for 5 min. The same conditions were maintained for the OaPV3, except for the annealing temperature at 57°C (Alberti *et al.*, 2010).

In OaPV3 reactions, a negative control (purified PCR-grade water) and a positive control (OaPV3 DNA cloned into pUC19 by Alberti and colleagues) were included. PCRs were repeated three times and the amplified products were visualized after electrophoresis in 2% agarose gel run containing GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, US) in TAE 1x buffer (40 mM Tris, 1 mM Na_2EDTA, 20 mM acetic acid), at 5 mVcm-1 for 90 min.

Table 2.1 Primer Sequences for Polymerase Chain Reaction

Primer	Sequence (5′–3′ orientation)	Product size, bp
L1 OaPV1 FP	CGCCCGTCTCCCTACGGTGC	177
L1 OaPV1 RP	CTGCAACGCCTCCGGACCCC	
L1 OaPV2 FP	CGCACCACAGCCCAAGGCAC	147
L1 OaPV2 RP	TCCAGCGTCCACACGGTCTGA	
L1 OaPV3 FP	AACTGGACTTGTCTTCCATG	127
L1 OaPV3 RP	AAAGACTCGGTATTGGGAGG	

Abbreviations: bp, base pairs; OaPV, *ovis aries* papillomavirus; FP, forward primer; RP, reverse primer

2.2.4 DNA in situ hybridization for OaPV3 localization in SCCs

In order to localize OaPV3 DNA in SCC samples, two digoxigenin-labeled and unlabeled probes have been generated by using PCR DIG Probe Synthesis Kit (Roche, Milano, IT), following the manufacturer's instructions. Specifically, a 151 base pair (bp) E6 DNA probe and a 127 bp L1 DNA probe were generated as previously described and used for colorimetric and fluorescence ISH experiments.[3]

A negative control (ovine liver) and a positive control (OaPV3 ISH-positive SCC sample), were included in each experiment. Briefly, deparaffinized and rehydrated tissue sections were placed in Tris-buffered saline (TBS) at 37 °C for 5 min, treated with 0.8% pepsin in HCl 0.2 N at 37 °C for 30 min, and rinsed in TBS. Subsequently, a post-fixation step was performed by dehydration in increasing concentrations of ethanol, from 70% v/v to 100%, and air-dried. Samples were treated with pre-hybridization solution [50% molecular grade hybridization solution II (Fluka Chimica, Milano, IT), 43% formamide, 7% mQ water] at 37 °C for 1 h, and then at 95 °C for 8 min and at 37 °C overnight with the hybridization solution containing the denatured

DNA probe [50% molecular grade hybridization solution II (Fluka Chimica, Milano, IT), 43% formamide, 6.5% of water and 10 ng/ml of E6 or L1 probes]. Subsequently, sections were washed in decreasing concentration of molecular biology grade SSPE [(Sigma-Aldrich, Milano, IT) 2x, 1x, 0.5x SSPE and 0.1x SSPE + BSA 0.2%], to remove the unbounded probes. After incubation for 1 h in blocking solution [2% normal rat serum, 0.3% Triton X-100 and TrisHCl (pH 8.2)], sections were incubated at 25 °C for 1 h with anti-digoxigenin-AP Fab fragments (Sigma-Aldrich, Milano, IT) diluted 1:200.

Color development was performed incubating sections at 37 °C for 50 min with detection buffer [TrisHCl 1M, NaCl 5M, MgCl2 1M (pH 9.5)] containing 2% 5-bromo-4-chloro-3-indolylphosphate and 2% 4-nitro blue tetrazolium chloride. After washing in mQ water, sections were closed with a coverslip using Top-Water mount (Sigma-Aldrich, Milano, IT).

Fluorescence developed sections were incubated in the dark at 25 °C for 45 min with Vector Red Alkaline Phosphatase Substrate Kit (Vector Lab, Burlingame, US). After washing in mQ water, nuclei were counterstained with Hoechst diluited 1:1000 and then sections were closed with Top-Water mount (Sigma-Aldrich, Milano, IT). Finally, slides were observed in a Leica TCS SP5 confocal microscope (Leica Microsystems, Germany) and the LAS AF Lite (Leica Microsystems) software was used for image processing.

The extent of colorimetric hybridization signal was evaluated based upon the brown nuclear signal, and semi-quantitatively scored according to the number of positive cells in 10 high power fields (400x) (grade 0: no positive cells; 1: 10-40%; 2: 41-70%; 3: >70.

2.2.5 Detection of OaPV3 early and late transcripts in SCCs

RNA from FFPE samples was extracted using the AllPrep DNA/RNA FFPE kit (Qiagen, Milano, IT). RT-PCR was performed using a two-step protocol. RNA was reverse transcribed (iScript cDNA Synthesis kit, Bio-Rad, Segrate, IT) following the manufacturer's instructions. PCR assays were conducted to detect OaPV3 early (E6) and late (L1) transcripts, and for the ovine GAPDH gene to assess cDNA quality (Alberti *et al.*, 2010; Budhia *et al.*, 2006). In each reverse transcription and amplification reaction, a negative control (purified PCR-grade water) and a positive control (OaPV3 RNA isolated from SCC tissue by Alberti and colleagues) were included. RT-PCRs were performed in a 50 µl master mix containing 1.5 mM $MgCl_2$, 0.2 µM of each oligonucleotide primer, 0.2 mM of each dNTP, 0.62 U of Taq DNA polymerase (Qiagen, Milano, IT) and 2.5 µl of cDNA.

Cycling conditions for E6 primers included initial denaturation at 95 °C for 5 min, followed by 40 cycles at 95 °C for 30 s, annealing at 52 °C for 30 s and extension at 72 °C for 30 s, with a final elongation step at 72 °C for 5 min. Reaction conditions were maintained for L1 primers, except for the annealing temperature at 57 °C.

RT-PCR results were visualized on 2% agarose gel containing GelRed™ Nucleic Acid Gel Stain (Biotium) after a run of 90 min in an electric field of 5 mVcm-1 in TAE 1x buffer.

A representative sub-set of amplified products positive for both OaPV3 E6 and L1 target were purified using the QIAquick Gel Extraction Kit (Qiagen, Milano, IT) and directly sequenced using the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Monza, IT). Sequenced fragments were analyzed by BLAST search of the NCBI GenBank database.

2.2.6 Statistical analysis

Statistical analyses were performed using Stata 11.2 software (StataCorp LP).

After performing descriptive statistics, categorical and ordinal variables were evaluated by Spearman rho (ρ) correlation coefficient, with Bonferroni adjustment and chi square ($\chi 2$ test) or Fisher's exact test. In particular, Spearman rank correlation was performed in order to assess the usefulness of the modified Anneroth's grading scheme in the histological evaluation of ovine SCC. A value of ρ approximately equal to 1 indicates a good correlation, a value near 0 indicates a poor correlation, and a negative value indicates an inverse correlation. Chi-square or Fisher's exact test was performed to assess the differences in PV presence in SCC and non-SCC samples, as well as the potential correlations between OaPV3 DNA presence in UV-exposed head regions (including eye, planum nasale and pinnae lesions) in the two samples set. A P value < 0.05 was considered significant.

2.3 Results

2.3.1 Histopathology

Grossly, SCCs appeared as horns (Fig 2.1 a) or cauliflower-like exophytic masses of variable size (from 4 to 9 cm) involving, in some cases, the dermal and sub-cutaneous structures, and frequently with epidermal ulceration (Fig 2.1 b).

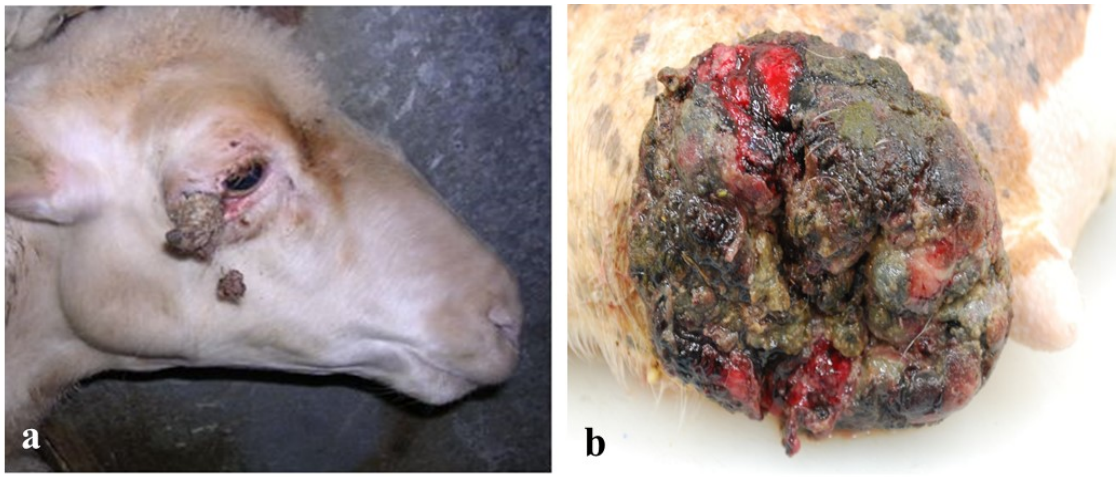

Fig 2.1 Ovine squamous cell carcinoma. **(a)** Macroscopical appearance of a cutaneous horn – like lesion localized in the eye of a sheep. **(b)** Macroscopical appearance of a cutaneous cauliflower – like lesion localized in the udder of a sheep.

Histologically, SCCs were characterized by cuboidal to polyhedral epithelial cell proliferations arranged in cords, trabeculae or islands. The majority of the examined neoplasms showed an orderly progression from polyhedral nonkeratinized cells localized at the periphery of the neoplastic epithelial islands, to large polygonal keratinized cells.

According to the modified Anneroth's multifactorial grading system, none of the SCC samples were well differentiated. Conversely, 36/40 (90%) SCCs localized in udder (N=19), eyelid (N=7), pinnae (N=5), planum nasale (N=3) and trunk (N=2), were moderately differentiated (median: 11; range 8-14). Histologically, moderately differentiated SCCs were characterized by trabeculae or small islands of squamous cells

associated with central eosinophilic keratin (keratin pearls) (Fig 2.2 a). Neoplastic cells exhibited prominent intercellular bridges, abundant amphophilic to eosinophilic cytoplasm and large, irregularly round to oval nuclei with finely stippled chromatin. Cells showed moderate anisocytosis and anisokaryosis and a variable number of mitoses ranging from 0 to 6 per HPF (1.52 0.20, mean ± SEM). Furthermore, a mild to moderate lymphoplasmacytic infiltrate intermingled with keratin pearls was frequently observed.

In contrast, 4/40 (10%) SCCs obtained from udder (N=2), planum nasale (N=1) and trunk (N=1) were poorly differentiated (median: 15; range: 15-20) and characterized by neoplastic cells organized in trabeculae rather than islands, disseminated in small groups or in keratinized single cells, often with invasion of the dermis (Fig 2.2 b). Cells showed extreme nuclear pleomorphism and the mitoses were atypical (mean 1.5 ± SE 0.20). Furthermore, a moderate lymphoplasmacytic infiltrate, admixed with degenerated neutrophils, scattered necrotic areas as well as stromal fibroplasia was observed.

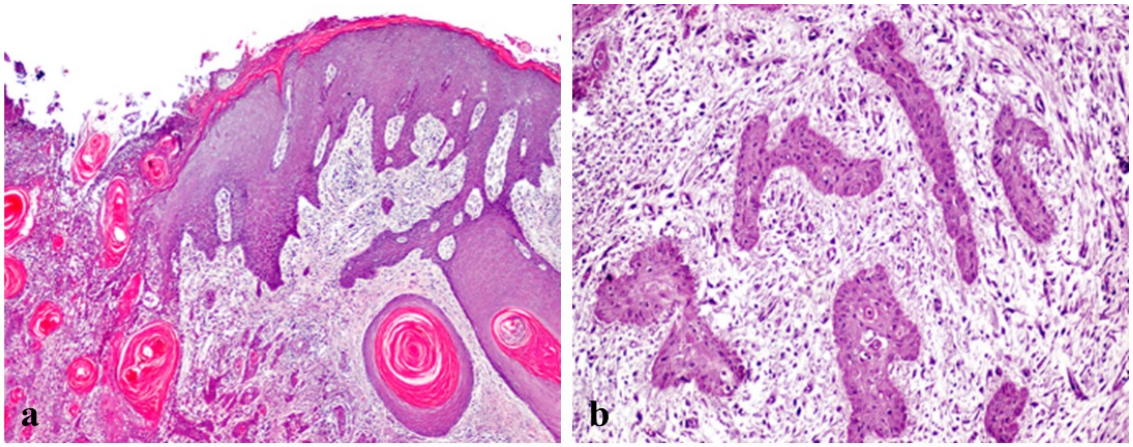

Fig 2.2 Ovine squamous cell carcinoma. **(a)** Histological features of a moderately differentiated SCC characterized by irregular cords of squamous cells with still evident keratin pearls (H&E). **(b)** Histological features of a poorly differentiated SCC characterized by islands of pleomorphic neoplastic cells embedded in an edematous stroma (H&E).

The superficial dermal collagen fibers of both SCC and non-SCC samples from eyelid, planum nasale and pinnae appeared pale and hypocellular (moderate solar elastosis) and, in certain cases, characterized by a mild degree of laminar fibrosis. In addition,

there was thickening of vessel walls with endothelial swelling and sometimes an obvious sclerotic change, often admixed with neutrophilic inflammation with a low number of lymphocytes, plasma cells and macrophages.

By Spearman rank correlation, the degree of keratinization was inversely associated with both nuclear pleomorphism ($\rho = -0.2348$, $P < 0.001$) and number of mitoses ($\rho = -0.1195$, $P < 0.01$) while, as expected, pleomorphism was positively associated with number of mitoses ($\rho = 0.2106$, $P < 0.001$) and pattern of invasion ($\rho = 0.3853$, $P < 0.001$). No statistical association was observed between the anatomic localization of the neoplasms and the SCC grade ($\chi2 = 4.15$, $P = 0.386$).

2.3.2 PCR detection of ovine PVs

OaPV3 DNA was amplified from 26/40 (65%) SCCs and 12/40 (30%) non-SCCs ($\chi2$ test = 9.82, P = 0.002, Table 2) (Fig 2.3). All examined samples tested negative for OaPV1 and OaPV2 DNA but were positive for the GAPDH gene. OaPV3 DNA was shown in 25/36 (69%) moderately differentiated SCCs localized in udder (N=12), eyelid (N=3), pinnae (N=5), planum nasale (N=3) and trunk (N=2), and in 1/4 (25%) poorly differentiated SCC inform the planum nasale. Similarly, OaPV3 nucleic acid was detected in non-SCC samples from the udder (N=6), eyelid (N=2), planum nasale (N=2) and from pinnae (N=1).

No statistical association was observed between the presence of OaPV3 and the SCC histological pattern (Fisher's exact = 0.139, P > 0.05), or between PV infection and the location of the SCC (Fisher's exact = 0.109, P > 0.05). Conversely, a higher OaPV3 positivity was observed in SCC samples from UV-exposed regions of the head (including eye, planum nasale and pinnae lesions) compared to non-SCC samples from these regions ($\chi2$ test =6.14, P =0.013).

2.3.3 Detection of OaPV3 by in situ hybridization

OaPV3 DNA was detected in 18/40 (45%) SCCs by colorimetric (Fig 2.4 a and b) and fluorescence ISH (Fig 2.5 a and b).

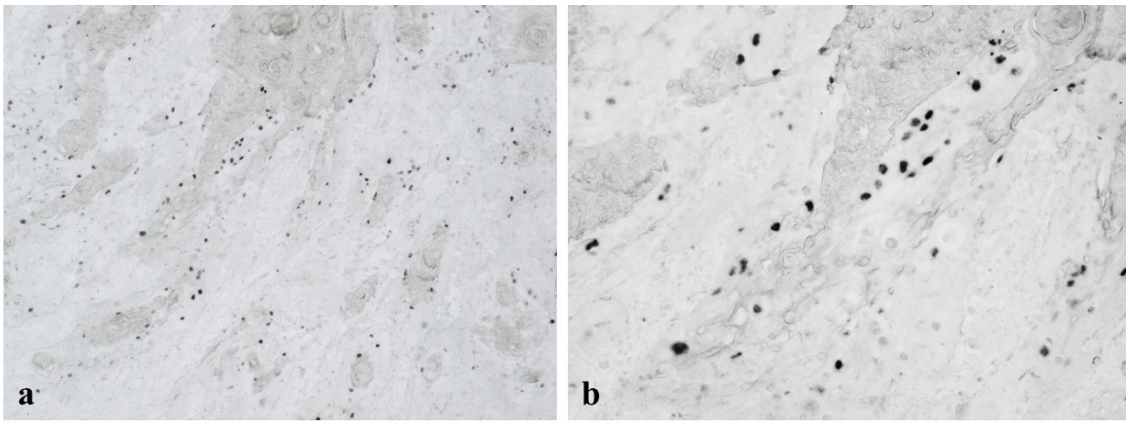

Fig 2.4 Colorimetric *in situ* hybridization of OaPV3 L1 probe. Hybridization signals detected in moderately differentiated squamous cell carcinoma sampled in pinnae. **(a)** High magnification. **(b)** Low magnification.

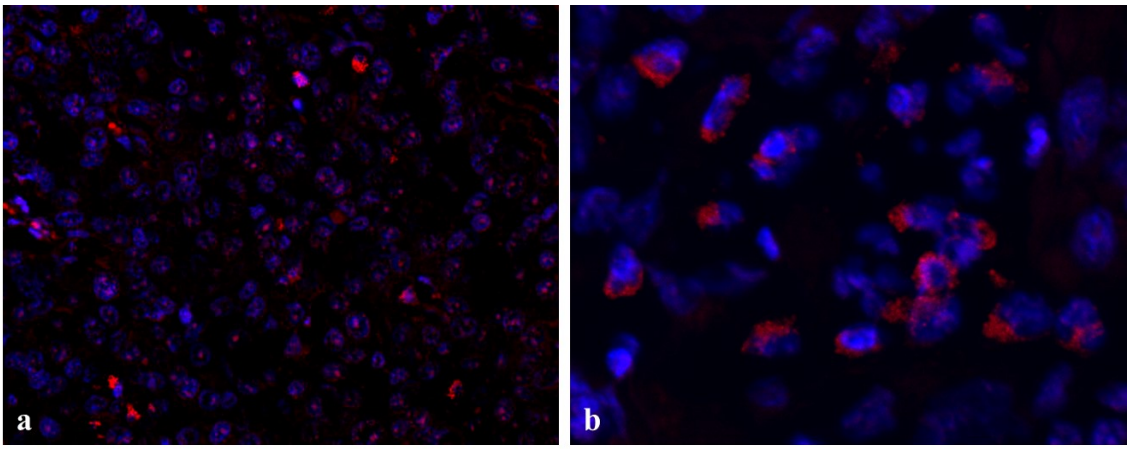

Fig 2.5 Fluorescence *in situ* hybridization of OaPV3 L1 probe. Hybridization signals detected in moderately differentiated squamous cell carcinoma sampled in eye. **(a)** High magnification. **(b)** Low magnification.

Specifically, the viral DNA was observed in 18/36 (50%) moderately differentiated SCCs from the udder (N=6), pinnae (N=4), eyelid (N=3), planum nasale (N=3) and

trunk (N=2). No viral DNA was detected in poorly differentiated SCCs (0/4) and in negative control slides.

Sixteen of 18 (89%) positive slides were scored as grade 1 (10-40% of positive cells), whereas 2 of 18 (11%) were scored as grade 2 (41-70% of positive cells).

Moderately differentiated SCC demonstrated strong and diffuse nuclear hybridization signals in malignant squamous cells mostly in the basal and intermediate and, less frequently, in the superficial layers of keratin pearls (Figs 2.6 and 2.7). In addition, a hybridization signal was detected in the hair follicular epithelium of one sample.

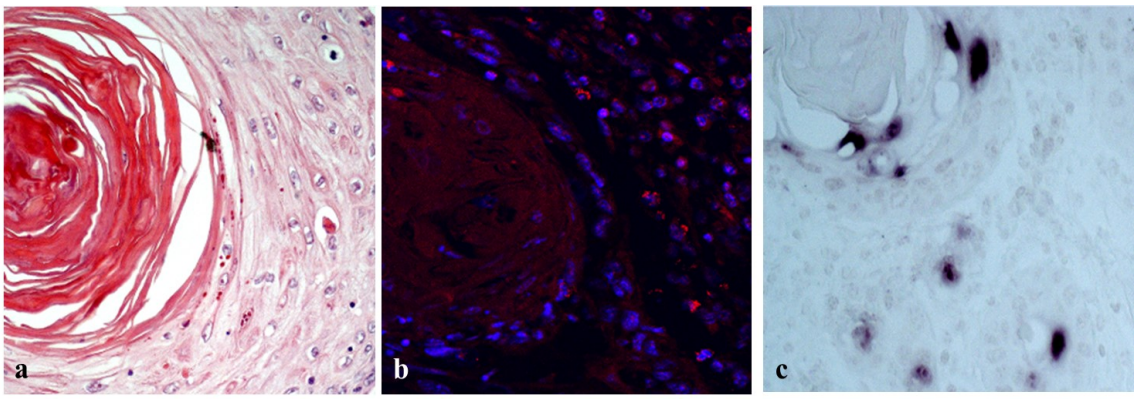

Fig 2.6 Moderately differentiated squamous cell carcinoma. **(a)** Histological aspect of a keratin pearl (H&E). **(b)** Fluorescence and **(c)** colorimetric OaPV3 hybridization signals in the nuclei of intermediate and superficial layers of moderately differentiated SCC sampled in pinnae.

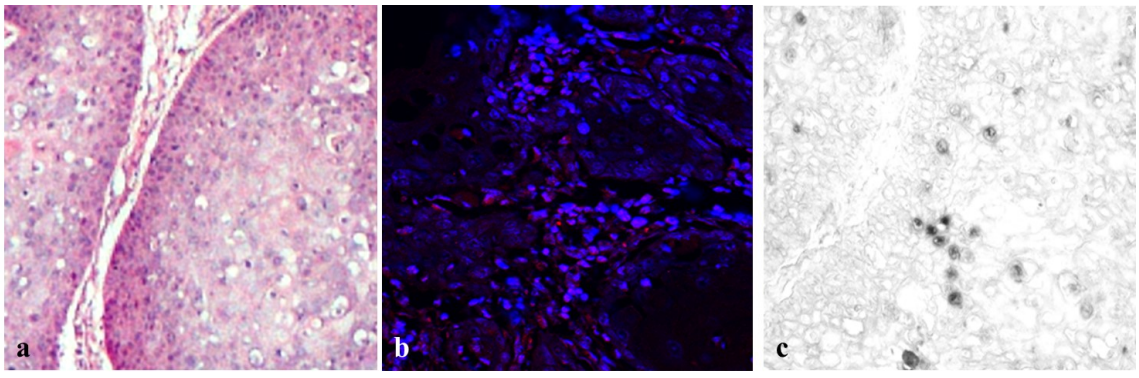

Fig 2.7 Moderately differentiated squamous cell carcinoma. **(a)** An island of malignant squamous cells (H&E). **(b)** Fluorescence and **(c)** colorimetric OaPV3 hybridization signals in the nuclei of a moderately differentiated SCC sampled in eye.

2.3.4 Detection of OaPV3 early and late transcripts in SCCs

OaPV3 early and late gene expression was detected by RT-PCR in 24/40 (60%) carcinomas. The presence of OaPV3 RNA, by RT-PCR, was identified in 23/36 (63%) moderately differentiated SCCs located in udder (N=10), pinnae (N=5), eye (N=3), planum nasale (N=3) and trunk (N=2), and in 1/4 (25%) poorly differentiated SCC, sampled in planum nasale (N=1). Specifically, 7/36 (19%) moderately differentiated SCCs revealed expression of both E6 and L1 genes, while 6/36 (16%) and 11/36 (30%) moderately differentiated SCCs revealed expression of only E6 or L1 transcripts, respectively (Fig 2.8 and 2.9). Finally, the poorly differentiated SCC exhibited only expression of the L1 gene. Amplification of the GAPDH gene was shown in all analyzed samples. The nucleotide BLAST search of the sequences confirmed a 100% identity for E6 and L1 OaPV3 transcripts.

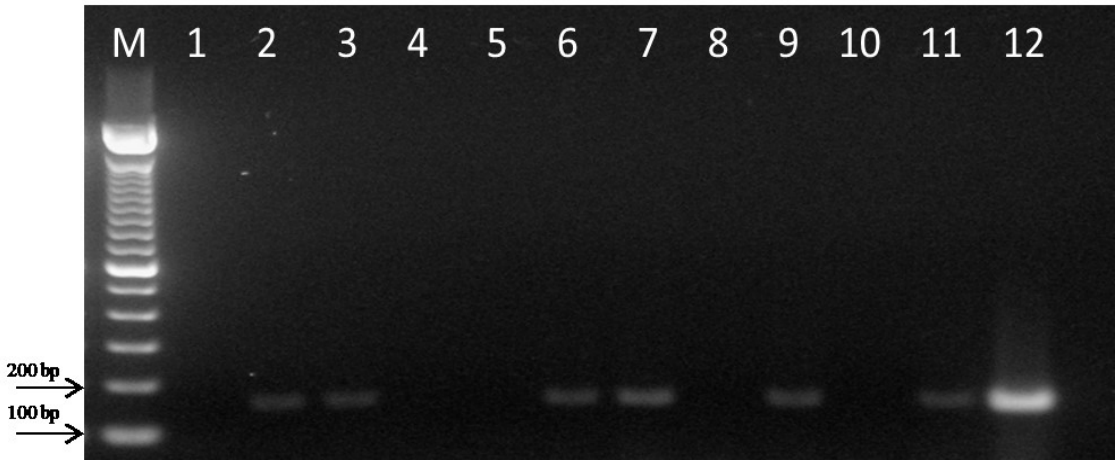

Fig 2.8 Agarose gel electrophoresis of OaPV3 E6 RT-PCR in squamous cell carcinoma samples. Six samples (lanes 2, 3, 6, 7, 9, 11) show the expected product size of 151 bp. M: molecular weight marker; lane 1: negative control; lane 2 – lane 11: udder samples; lane 12: positive control.

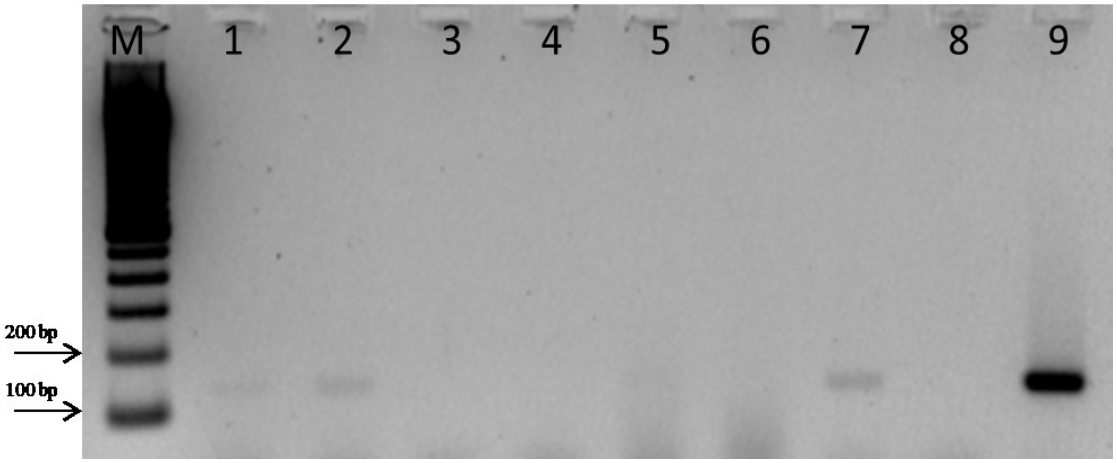

Fig 2.9 Agarose gel electrophoresis of OaPV3 L1 RT-PCR in squamous cell carcinoma samples. Three samples (lanes 1, 2, 7) show the expected product size of 127 bp. M: molecular weight marker; lane 1 – lane 7: udder samples; lane 8: negative control; lane 12: positive control.

Overall, PCR detected OaPV3 DNA in 26/40 (65%) SCCs, whilst RT-PCR revealed the presence of cDNA in 24/40 (60%) SCCs. Notably, these two RT-PCR negative samples, were OaPV3 positive by both PCR and ISH.

2.4 Discussion

In this report we evaluated the presence of ovine PV DNA within SCC and non-SCC samples, by PCR using specific primers for OaPV1, OaPV2, OaPV3. As result, a high prevalence of OaPV3 DNA (65%) was identified in SCCs of Sardinian sheep. Furthermore, as previously reported[3], a lower prevalence (30%) of OaPV3 DNA in non-SCC samples was also observed, suggesting a possible etiological role for the virus in the tumor onset as proposed in humans (Chen *et al.*, 2008; Alberti *et al.*, 2010). Conversely, none of the tested samples showed the presence of OaPV1 or OaPV2 DNA. This could be related to the genome organization of these viruses. In fact, OaPV1 and OaPV2 encode the E5 hydrophobic transmembrane protein, a well characterized protein in bovine PV 1 with a strong transforming activity in fibroblasts (Bocaneti *et al.*, 2016). For this reason, and considering also the high sequence homology between the E5 genes of the Delta genus PVs types, we could speculate that OaPV1 and OaPV2 are involved only in fibropapilloma tumor development (Borzacchiello and Roperto, 2008). Conversely, OaPV3 shows an epithelial tropism and lacks the E5 gene, but retains E6 and E7 protein, involved in several functions such as anoikis, invasion and altered functions of the p53 oncoprotein (Munger *et al.*, 1989; Narisawa-Saito and Kiyono, 2007; Alberti *et al.*, 2010; Corteggio *et al.*, 2013). In addition, OaPV-3 encodes the conserved retinoblastoma (Rb) tumor suppressor binding sequence motif in E7 that, as reported by Munger (1989) and Bourgo (2009), promotes uncontrolled cell division by forming stable complexes with the Rb codified protein (Munger *et al.*, 1989; Bourgo *et al.*, 2009). These data, and the higher amplification rate in SCC samples compared to non-SCC samples, suggest that OaPV3 may play a preferential key role in epithelial malignant tumor development.

OaPV3-specific nuclear signal was detected in 45% of ovine SCCs by ISH technique. Even though PCR, as expected, was more sensitive in detection of OaPV3 DNA, and indeed revealed higher positivity than ISH, ISH is advantageous for localization and quantification of infected cells (Hayat, 2004).

Colorimetric ISH was most useful to detect the virus in tumor cells, while the nuclear signals were more marked by fluorescence analysis compared to colorimetric method. The localization of OaPV3 nucleic acid was revealed in malignant squamous epithelial cells, confirming the epithelial tropism of this PV (Alberti *et al.*, 2010).

Furthermore, in one case (case No. 29) the virus was detected in hair follicle epithelium. This result is in agreement with previously and recently reported data that support the hypothesis that adnexa could represent an important site of entry of the virus and, consequently, a reservoir for many PV types (Munday and Kiupel, 2010; Doorbar *et al.*, 2012; Gaynor *et al.*, 2016). As previously suggested, additional and focused studies will be necessary to prove this hypothesis (Gaynor *et al.*, 2016).[24]

RT-PCR for early and late genes showed the OaPV3 transcriptional activity in 60% of SCCs, similar to reports in human and cattle (Borzacchiello *et al.*, 2003; Ukpo *et al.*, 2011; Bishop *et al.*,2012). Additionally, based on gene expression pattern, RT-PCR showed different stages of the viral life cycle, corroborating the hypothesis of a productive infection in tumor cells.

Through the RT-PCR analysis, OaPV3 cDNA was found in 24 of 26 PCR positive SCCs. Furthermore, the presence of viral DNA with the concomitant lack of transcriptional activity in two cases (case Nos. 1 and 5, despite amplification of the ovine GAPDH confirming RNA integrity) could indicate a silent PV infection (Campo *et al.*, 1994; Maran *et al.*, 1995). These results are in agreement with previously reported data in humans and cattle, in which the activated host immune system is able to restrict

the PV infection in basal cells, leading to altered transcriptional activity, with the lack of E6 and L1 viral gene expression in immunocompetent animals (Campo *et al.*, 1994; Maran *et al.*, 1995; Stubenrauch and Laimins, 1999; Doorbar, 2005).

Our results showed that the modified Anneroth's multifactorial grading system could be useful to characterize ovine cutaneous SCC, as confirmed by the statistical and biological relationship between parameters such as cellular pleomorphism, number of mitosis and degree of invasion. The prognostic value of this grading system needs to be confirmed by additional studies. The different grades of ovine SCC were unrelated to PV infection, suggesting (as proposed in humans) that PVs are not the only factors responsible for tumor onset (Forslund *et al.*, 2007; Ally *et al.*, 2013). In particular, the higher OaPV3 positivity in SCC samples from the UV-exposed regions compared to matched non-SCC samples suggests that the virus could represent a synergic factor together with prolonged exposure to ultraviolet radiation and poor skin pigmentation in tumor development and onset. Likewise, as demonstrated by in vitro studies, the local UV immunosuppressive effect promotes cellular malignant transformation stimulating the activity of PV and increasing the viral protein levels (i.e E6) able to suppress pro-apoptotic proteins (Purdie *et al.*, 1999; Akgul *et al.*, 2006)

2.5 Conclusion

This study reports the first investigation of OaPV3 infection in a large number of Sardinian sheep with SCC and offers new insight into the pathogenesis of squamous cell carcinoma in small ruminants. Our data supports a multifactorial etiology for the development of SCC and suggests that the pathogenesis for ovine SCC may involve a range of complex interactions, including PV infection.

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Chapter III

Biomarker discovery of *Ovis aries* papillomavirus 3 in ovine cutaneous squamous cell carcinoma

3.1 Introduction

The field of proteomic includes the large-scale study of proteins, including their structures and functions, to comprehensively map biological processes (Alvarez-Chaver *et al.*, 2014). The set of proteins expressed by the genetic material of an organism under defined environmental conditions is referred as proteome (Ceciliani *et al.*, 2014). The proteome study is a complex process due to the activation of multiple pathways involved in the synthesis of the native proteins, such as alternative transcription initiation, alternative splicing RNA, proteolytic processing and post-translational modifications (Alvarez-Chaver *et al.*, 2014). Based on these considerations, several approaches have been performed in order to identify candidate protein markers that are differentially expressed under specific conditions. Two-dimensional electrophoresis (2-DE) and two-dimensional differential in-gel electrophoresis (2D-DIGE) techniques are routinely used for the differential proteins identification, while surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) and the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) are used in order to determine a minute protein profile differences (Cadieux *et al.*, 2004; Viswanathan *et al.*, 2006). Furthermore, more recently, the liquid chromatography-tandem mass spectrometry (LC-MS/MS), commonly referred as shotgun analysis, has been widely used for more sensitivity analysis in which proteins from a complex mixture are collectively in-solution digested and separated by high-performance liquid chromatography (HPLC). Then, chromatographic fractions are analyzed by a tandem mass spectrometer, allowing the isolation and the fragmentation of individual peptides for proteins identification (Karpievitch *et al.*, 2010).

The recent revolution in proteomic profiling technologies allowed the discovery of specific biomarkers, representing sensitive and specific molecular tools particularly useful in cancer research (Mabert *et al.*, 2013). A widely used definition of a biomarker is "an objectively measured characteristic, describing a normal or abnormal biological state, detectable by the analysis of biomolecules such as DNA, RNA, protein and peptide". Biomarkers may be detected in tissue, in circulation, and in body fluids. They have many important potential roles in clinical practice, leading to the prediction of response to therapy, the prediction of clinical outcome, risk assessment, screening, diagnosis and pharcogenetics. Based on these application, biomarkers can be broadly classified into three categories. Diagnostic biomarkers are able to identify a specific disease condition, while prognostic biomarkers provide important information regarding the risk of clinical outcomes such as cancer recurrence or disease progression. Finally, predictive biomarkers predict response to specific therapeutic interventions (Goossens *et al.*, 2015).

The identification of altered protein occurring in oncogenesis process, as well as their qualitative and quantitative characterization, can offers valuable information relating to more effective diagnosis, prognosis, and response to therapy. In the last few years, the application of several proteomics approaches in human medicine provided useful insight, enabling the molecular characterization of various human diseases and the identification of specific molecular mechanism dictating the initiation and progression of the disease. The proteomics studies of the abundance proteins changes between different cellular state provided insight into the pathophysiological basis of protein target in the intervention and treatment disease (Lippolis and De Angelis, 2016).

The application of proteomics in veterinary medicine has been limited in comparison to human. Nevertheless, as previously suggested by Ceciliani and colleagues (2014), the

increase in biomarkers identification is urgently needed in veterinary medicine for diagnosis and prognosis of disease.

Only recently, an increased activity of animal proteomic studies has been observed in literature, especially for investigation of farm animal health and disease (Ceciliani *et al.*, 2014). Nevertheless, unlike cows and pigs, most of proteomic research on sheep has been focused on parasites related to gastrointestinal infections and none studies investigating differential proteins related to cancer have been reported. The application of proteomic analysis in ovine cutaneous squamous cell carcinoma (SCC) appears thus particularly interesting in order to map the biological process involved in the etiology of this tumor, in which the infection of *Ovis aries* papillomavirus 3 (OaPV3) seems to play a central role. The identification of protein-related to molecular mechanisms involved in tumor pathogenesis can represent an important tool for the knowledge of pathways related to the OaPV3 infection, as well as for the identification of candidate biomarkers related to the viral activity. Based on these consideration, the aim of the present study was to discover and validate OaPV3 infection-related proteins in ovine cutaneous SCC, by proteomic and immunohistochemical analysis.

3.2 Materials and Methods

3.2.1 Origin of the samples

Thirty cutaneous samples obtained from udder and head region of Sardinian sheep were selected from our previous analysis and classified into three groups, including 10 non-SCC samples, 10 OaPV3 negative SCCs and 10 OaPV3 positive SCCs.

Specimens were divided into two aliquots and stored in appropriate conditions based on the downstream analysis to be performed. For histological examination, 10% formalin-fixed and paraffin embedded (FFPE) samples were 3 μm-sectioned and stained with hematoxylin and eosin (H&E). For protein extraction, complementary tissues were snap frozen and stored at -80°C until use. A subset of representative samples (9 samples from udder), including 3 non SCC samples, 3 OaPV3 negative SCC and 3 OaPV3 positive SCC sections, were included in the analysis. Each sample was embedded inOptimal Cutting Temperature medium (Tissue-Tek®, Sakura Finetek, Torrance, CA, USA) and 20 frozen section (10 μm tick) were sectioned on a Leica cryotome Cryostat (Leica CM 1950, Heidelberg, Germania) and collected in 1.5 ml tubes.

3.2.2 Tissue protein extraction and SDS-PAGE

Proteins were extracted by incubating frozen sections in 100 μl of a buffer containing 2 % sodium dodecyl sulfate (SDS), 0.4% Tween-20, 130 mM dithiothreitol (DTT) and 500 mM Tris-HCl (pH 8.8), plus SIGMAFAST™ Protease Inhibitors (Sigma, St. Louis, MO, USA) at the concentration recommended by the manufacturers, for 15 min at 95 °C at 300 rpm using a Thermomixer comfort (Eppendorf, Hamburg, Germany). After centrifugation at maximum speed, the supernatants containing proteins were quantified using Pierce^{TM} 660 nm Protein Assay (Thermo Scientific). Then, 15 μg of each protein

extract was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) in a 4-20 % Mini-PROTEAN TGX^{TM} Precast Gel (Biorad), which was stained with Simply Blue Safe Stain (Invitrogen, Carlsbad, CA, USA).

3.2.3 Shotgun analysis

The gel regions corresponding to different molecular weight (MW) were excised, destained and trypsin digested by using Filter Aided Sample Preparation (FASP) in a 10 kDA Amicon Ultra-0.5 ml Centrifugal Filters (Millipore, Merck), as previously described (REF). Briefly, 100 µg of proteins were mixed with 500 µl of 8 M UA solution containing 8 M urea in 100 mM Tris-HCl (pH 8.8), loaded onto the 10 kDA Amicon Ultra-0.5 ml Centrifugal Filters, and centrifuged at 12.000 x g for 25 min at 20°C.The concentrates were diluted in the devices with 500 µl UA solution and centrifuged again. Next, the concentrates were mixed with 200 µl of 10 mM DTT in 8 M UA solution and incubated in the dark for 30 min at room temperature (RT). Then, the concentrate was diluted with 200 µl of 50 mM iodoacetamide in 8 M UA solution, incubated in the dark for 20 min at 20°C and centrifuged at 12.000 x g for 25 min at 20°C. This step was repeated for 3 times. After the flowthrough was discarded, 500 µl of 50 mM ammonium bicarbonate was added to the filter and centrifuged at 12.000 x g for 15 min; this step was repeated 3 times. Proteins were then digested at 37°C overnight using trypsin (enzyme to substrate ratio [w/w] of 1:100), centrifuged at 12.000 x g for 25 min at 20°C and eluted in a new collection tubes. This step was repeated 2 times and the eluted peptides were resuspended in 0.2 % formic acid. Then, liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) analyses of tryptic digests were performed on a Q-TOF hybrid mass spectrometry (Waters). Each sample was analyzed in duplicate.

3.2.4 Protein identification and quantification

Proteome Discoverer Mass Informatics Platform (version 1.4; Thermo Scientific) was used for protein/peptide identification, using Sequest-HT as search engine (*Protein Database:* UniProtKB/SwissProt with *Bos Taurus, Ovis aries, Capra hircus and Papillomaviridae* as taxonomy; *Enzyme*: Trypsin; *Maximum missed cleavage sites:* 2; *Precursor mass tolerance:* 50 ppm; *Fragment mass tolerance:* 0.4 Da; *Static modification:* cysteine carbamidomethylation; *Dynamic modification:* N-terminal Glutamine conversion to Pyro-glutammic acid and methionine oxidation), and Percolator for peptide validation (peptide confidence: q-value<0.01).

Spectral counts (SpC) were used for estimating protein abundance and comparing the expression of the same protein between samples. SpC log Ratio (Rsc) and Normalized Spectral Abundance Factor (NSAF) were calculated in order to estimate the proteins fold change between different conditions and the relative abundance, respectively. A principal components analysis (PCA) was additionally performed by using the NASAF value, in order to evaluate the proteomic profile in each samples group.

Finally, dysregulated proteins were subjected to the gene ontology (GO) bioinformatics analysis by using BLAST and STRING database for enrichment categories of functional annotation, networks and diseases-related proteins.

3.2.5 Statistical analysis

The statistical significance of Rsc was evaluated by using the beta-binomial test ($P \leq 0.05$), with the false discovery rate (FDR) correction factor. Similarly, NSAF results was evaluated by using t-test ($P \leq 0.05$)

3.2.6 Validation of deregulated proteins by immunohistochemistry

To analyze the deregulated proteins, histological sections (3 µm tick) from FFPE samples were mounted on charged slides (Superfrost Ultra Plus, Thermo Scientific). After tissues dewaxing and rehydration, antigens were retrieved by a preheated citrate solution (pH 6.6) (BiOptica, Milan, Italy) for 20 min at 98°C. The slides were cooled at room temperature for 20 min, and tissues were blocked for endogenous peroxidase with Dako REAL Peroxidase-Blocking Solution (S2023, Dako, Glostrup, Denmark). Then, sections were blocked for non-specific binding in 2% bovine serum albumin (BSA) and in 2.5% normal horse serum (ImmPRESS reagent kit,Vector Labs, Burlingame, CA, USA) for 1 h at room temperature. Subsequently, slides were incubated overnight at 4°C with rabbit polyclonal antibodies against 14-3-3 theta protein (BML-SA481) and cytokeratin 13 (ab58744) at 1:2500 and 1:400 dilution, respectively. Then, slides were incubated with an anti-rabbit secondary antibody (ImmPRESS reagent kit,Vector Labs, Burlingame, CA, USA) for 30 min at room temperature. After staining with 3,3'-Diaminobenzidine (ImmPACT DAB, Vector Laboratories, Burlingame, CA, USA) tissues were counterstained with hematoxylin, cover-slipped with Eukitt Mounting Medium^{TM} (BiOptica, Milan, Italy) and observed under light microscopy. Control slides of brain and urinary bladder were incorporated into each experiment as positive controls of the 14-3-3 protein and cytokeratin 13, respectively. Negative controls were carried out by replacing the primary antibody with normal rabbit serum (Invitrogen, Milan, Italy).

A semi-quantitative score based on the stain intensities was applied in order to evaluated the expression of selected proteins.

3.3 Results

3.3.1 Proteins extraction and SDS-PAGE

The quantification of protein extracts by using Pierce™ 660nm Protein Assay showed a good concentration in each samples, as reported in Table 3.1 and in Fig 3.1. Additionally, each group of protein extracts separated by SDS-PAGE exhibited a good reproducibility. As showed in Fig 3.2, a strong overlap between the proteins set of each group was observed.

Samples Group	Sample	Concentration (mg/ml)
non-SCC samples	1	1,484
	2	1,454
	3	1,429
OaPV3 negative SCCs	4	1,549
	5	1,379
	6	2,100
OaPV3 positive SCCs	7	2,360
	8	2,876
	9	1,344

Table 3.1 Quantification of protein extracts. The protein concentrations of each samples are showed in mg/ml.

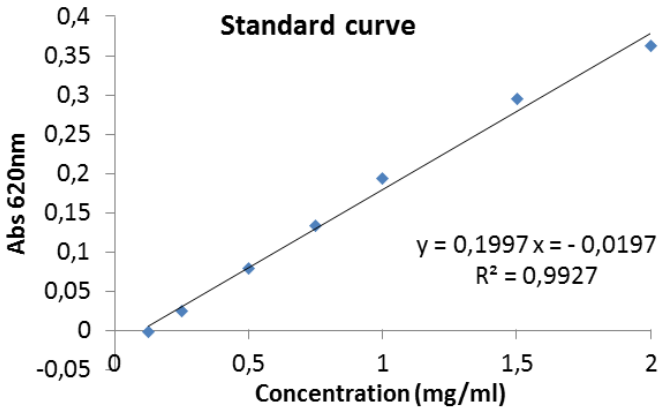

Fig 3.1 Standard curve of samples concentration.

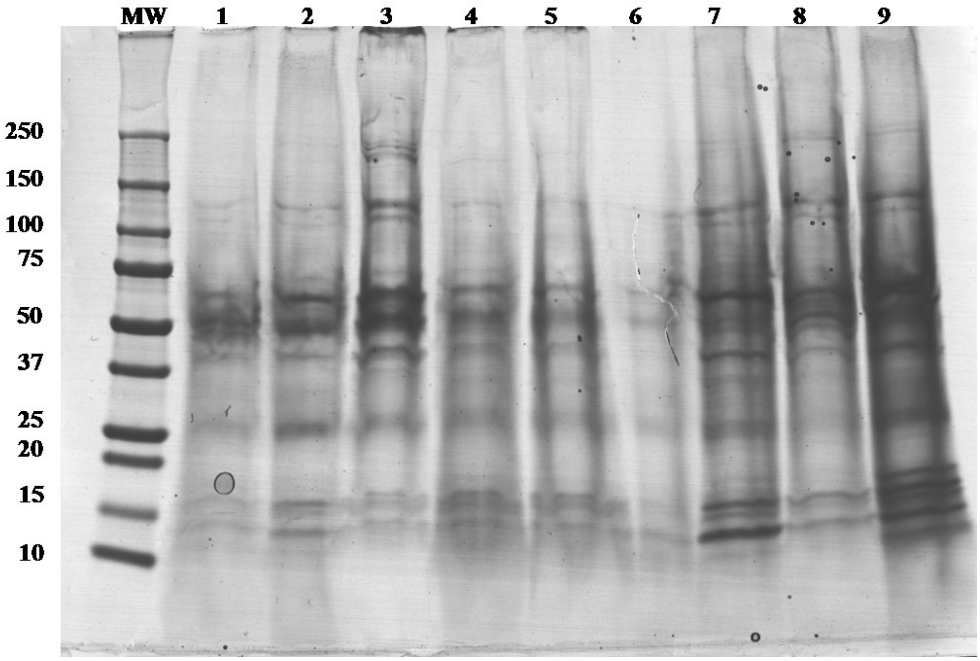

Fig 3.2 SDS-PAGE of tissue protein extracts. Lanes 1-3: OaPV3 negative SCCs; Lanes 4-6: OaPV3 positive SCCs; Lanes 7-9: non-SCC samples; MW: molecular weight marker. Standard MWs are displayed on the left side.

3.3.2 Protein identification and multivariate analysis

Results identified 476 proteins, of which 242 were included in the differential analysis between the three groups. The multivariate statistical analysis by PCA based on the NSAF values was used to examine the global trends in protein expression of the three groups. These samples were grouped according to the variance of their protein expression (% V) and their spatial distribution is shown in Fig 3.3. The first principal component explained 27.3% of the variance and the third explained a further 13.8%. PCA showed that samples clustered in three distinct groups, in which only one sample in both non-SCC samples and OaPV3 negative SCCs exhibited differential characteristics. Furthermore, non-SCC samples and OaPV3 negative SCCs exhibited more close clusters, if compared to OaPV3 positive SCCs, indicating quantitative and qualitative differences in the OaPV3 positive SCCs group.

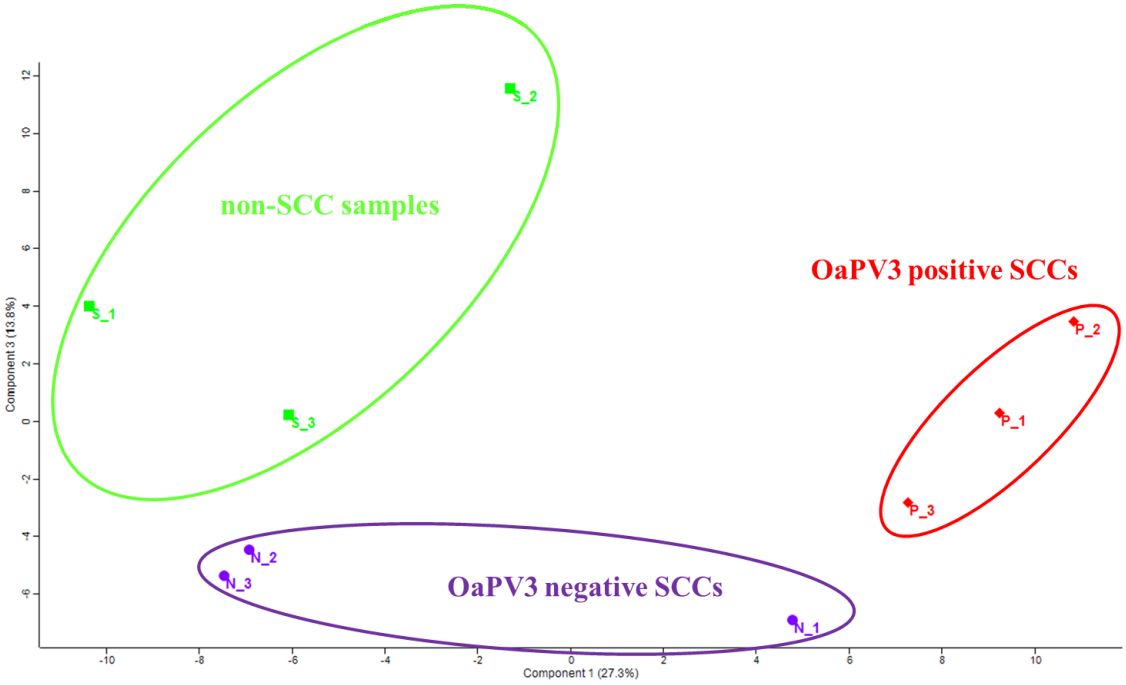

Fig 3.3 PCA based on the NSAF values between the three groups.

These differences were confirmed by the differential analysis of proteins profile between the three groups. In this analysis, proteins showing a Rsc value between -1.5≤Rsc≥1.5 and p-value≤0.05 were considered differential proteins. In particular, 42 proteins were identified as differentially expressed between OaPV3 positive SCCs and non-SCC samples, of which 13 up regulated and 29 down regulated in OaPV3 positive SCCs, as showed in Fig 3.4.

Twenty-six differential proteins were detected between OaPV3 negative SCCs and non-SCC samples, of which 7 were up regulated and 19 down regulated in OaPV3 negative SCCs (Fig 3.5). Finally, 28 differential proteins were detected in OaPV3 negative SCCs and OaPV3 positive SCCs, of which 17 up regulated and 11 down regulated in OaPV3 positive SCCs (Fig 3.6).

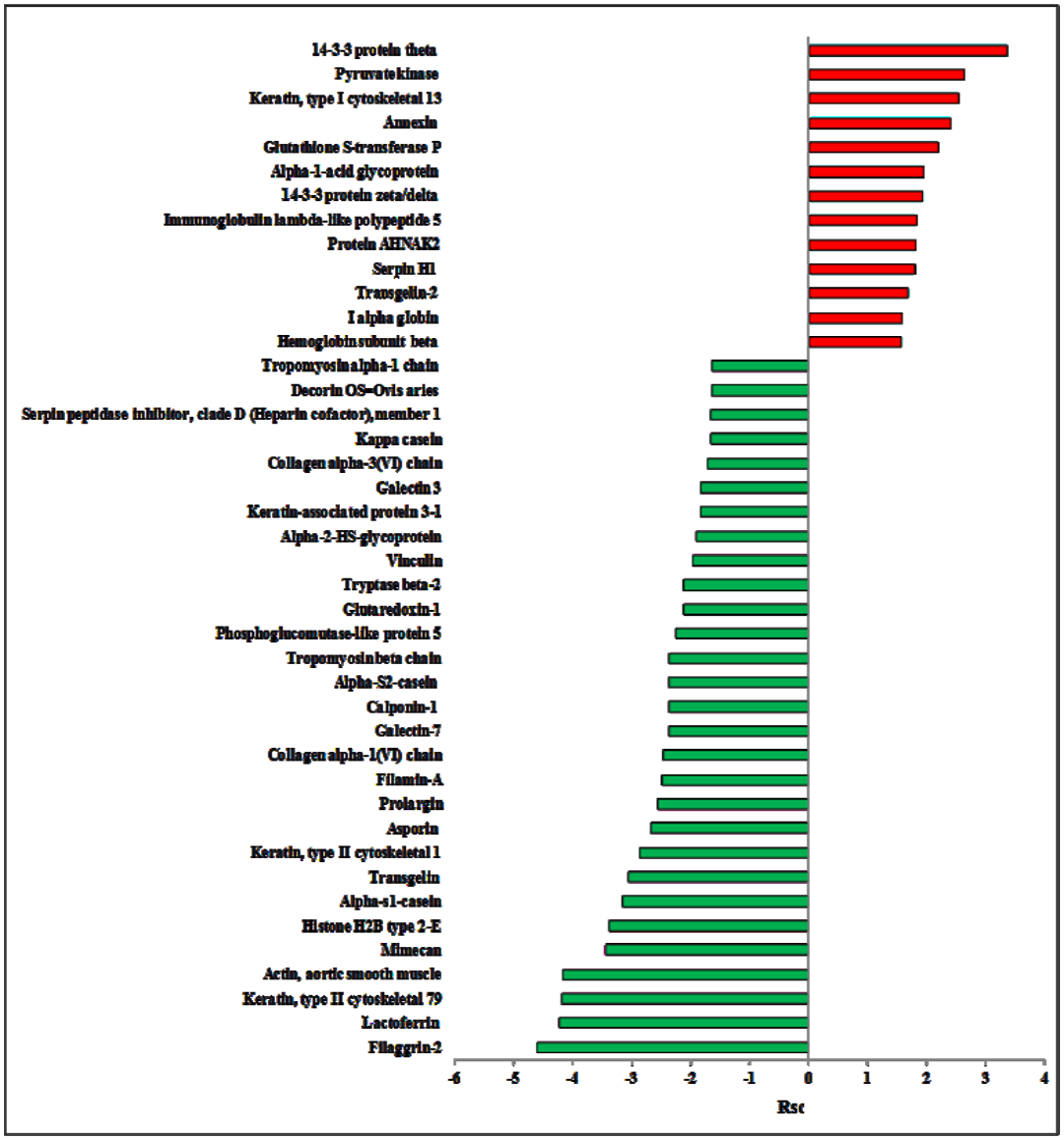

Fig 3.4 Rsc of differential proteins between OaPV3 positive SCCs and non-SCC samples. Up regulated proteins are indicated in red, while down regulated proteins are showed in green.

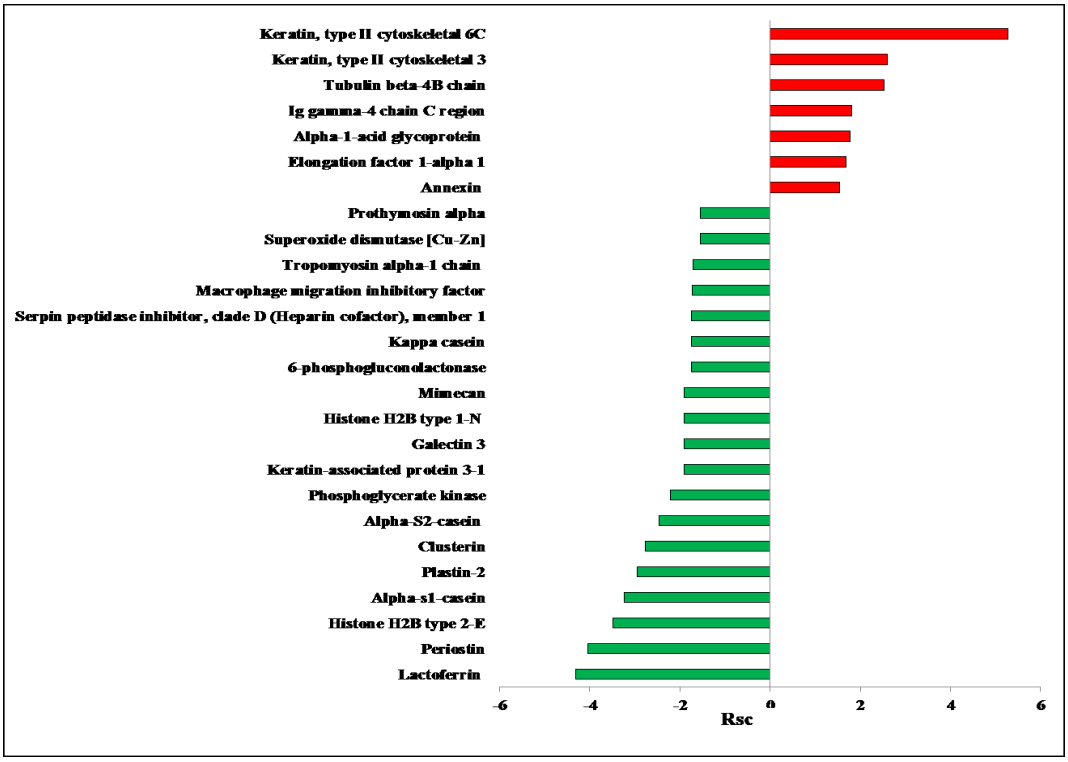

Fig 3.5 Rsc of differential proteins between OaPV3 negative SCCs and non-SCC samples. Up regulated proteins are indicated in red, while down regulated proteins are showed in green.

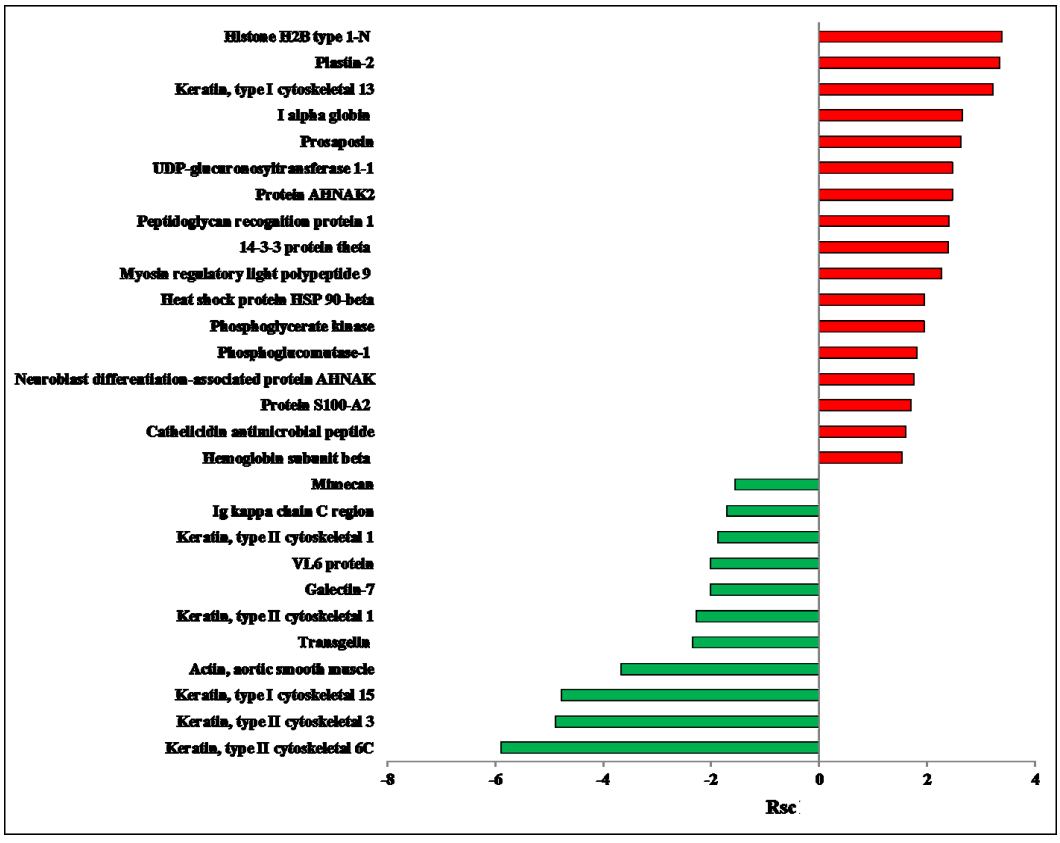

Fig 3.6 Rsc of differential proteins between OaPV3 positive SCCs and OaPV3 negative SCCs. Up regulated proteins are indicated in red, while down regulated proteins are showed in green.

Differential proteins between the three groups were then assembled by STRING and BLAST networks, in order to evaluate the biological processes and molecular functions in which proteins are involved. STRING network results are showed in Fig 3.7. Response to stress, regulation of apoptotic signaling pathway, negative regulation of apoptotic process and cell death, tissue development and epithelial cell differentiation, as well as extracellular matrix disassembly and organization, and glycosaminoglycan catabolic process were the principal biological processes associated to the differential proteins. A significant increase of processes related to stress, regulation of apoptotic signaling pathway, negative regulation of apoptotic process and cell death were identified in OaPV3 positive SCCs compared to OaPV3 negative SCCs and non-SCC samples. Conversely, a significant decreased of tissue development, epithelial cell differentiation, extracellular matrix disassembly and organization, and glycosaminoglycan catabolic process were observed in OaPV3 positive SCCs and non-SCC samples.

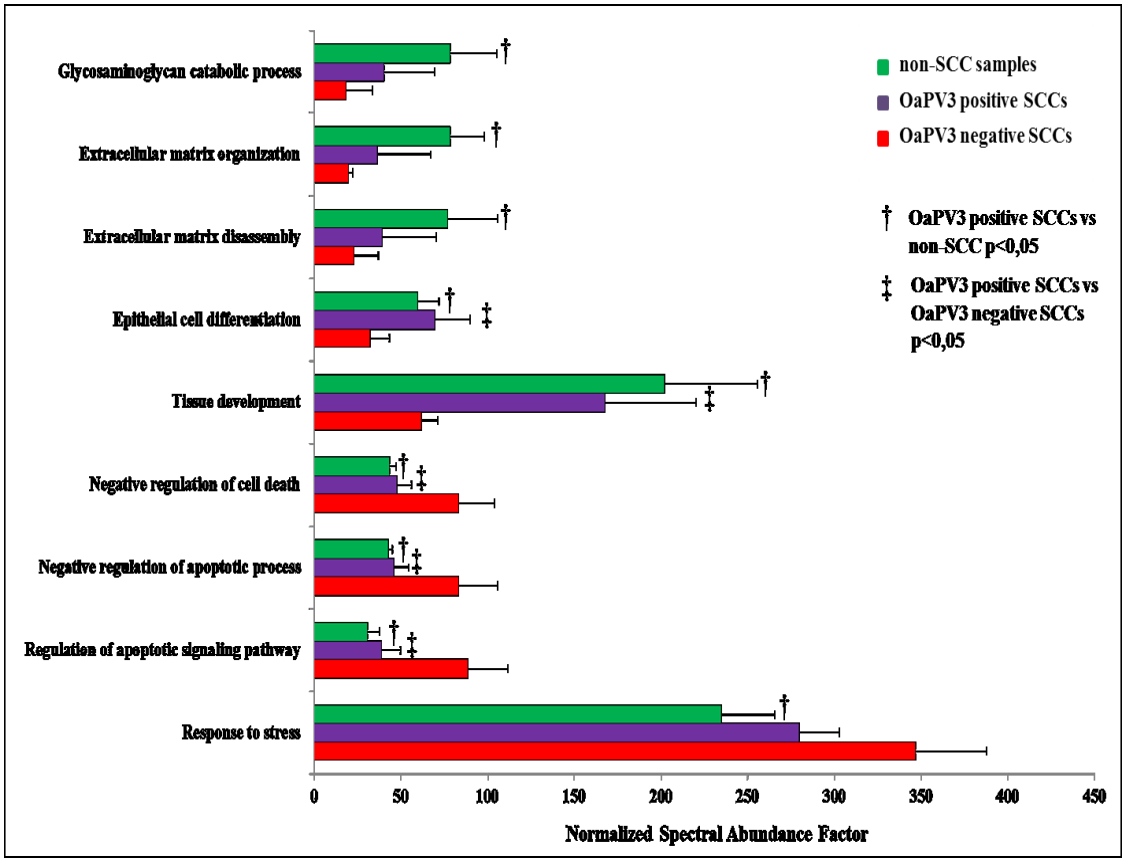

Fig 3.7 Abundance of biological processes related to differential proteins showed in OaPV3 positive SCCs (red), OaPV3 negative SCCs (violet) and non-SCC samples (green).

3.3.3 Immunohistochemical validation of 14-3-3 theta protein

The 14-3-3 theta immunohistochemical result was unexpected. In fact, a strong immunoreactivity signal was detected in both nuclei and cytoplasm of malignant and normal epidermal cells, as well as in most of dermal structures including fibroblasts, vessel endothelial cells and adnexa structures. These results were consistently observed in examined OaPV3 positive and negative SCCs, as well as in normal skin tissues. Furthermore, non-specific signals were also detected in brain samples suggesting thus additional antibody-epitope binding in sheep tissues.

3.3.4 Immunohistochemical validation of cytokeratin 13

The overexpression of cytokeratin 13 was confirmed in examined OaPV3 positive tissues by immunohistochemistry. In particular, 10/10 OaPV3 positive SCCs shown diffuse cytoplasm signals. The immunoreactivity were mostly observed in the intermediate and superficial layers of keratin pearls, while signals were rarely observed in the epithelial basal layer (Fig 3.8). In particular, 6/10 OaPV3 positive SCCs shown a strong immunoreactivity signal, whereas 4/10 samples shown a weak immunostaining (Fig 3.9; Table 3.2). Furthermore, only 2 out of 10 OaPV3 negative SCCs shown a barely perceptible signal in suprabasal cells, whilst no signals were detected in 8/10 and 10/10 OaPV3 negative SCC (Fig 3.10 a and b; Table 3.2) and non SCC samples (Fig 3.10 c and d; Table 3.2), respectively.

A strong immunoreactivity was detected in the cytoplasm of bladder epithelial cells used as positive controls, while no signal was observed in the negative controls slides (Fig 3.10 e and f).

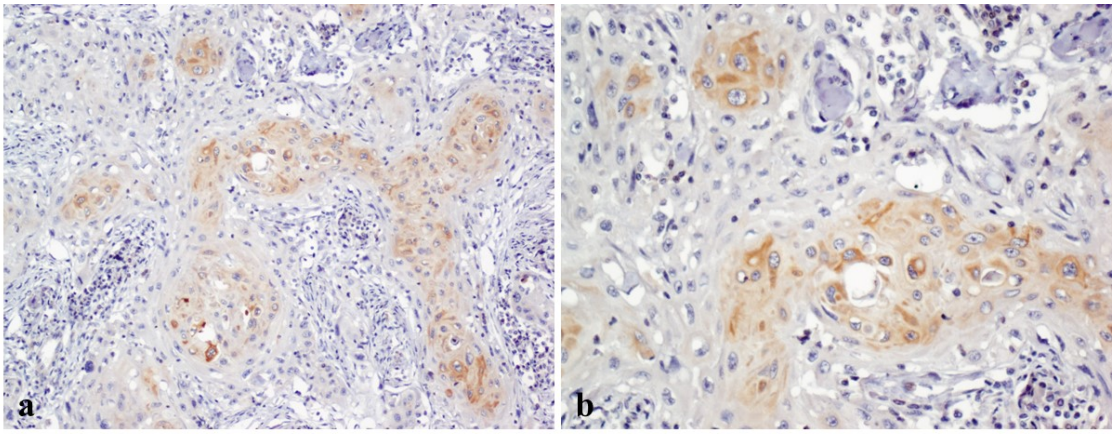

Fig 3.8 Immunohistochemistry of cytokeratin 13. **(a)** Low and **(b)** high magnification of immunoreactivity signals detected in the intermediate and superficial layers of keratin pearls.

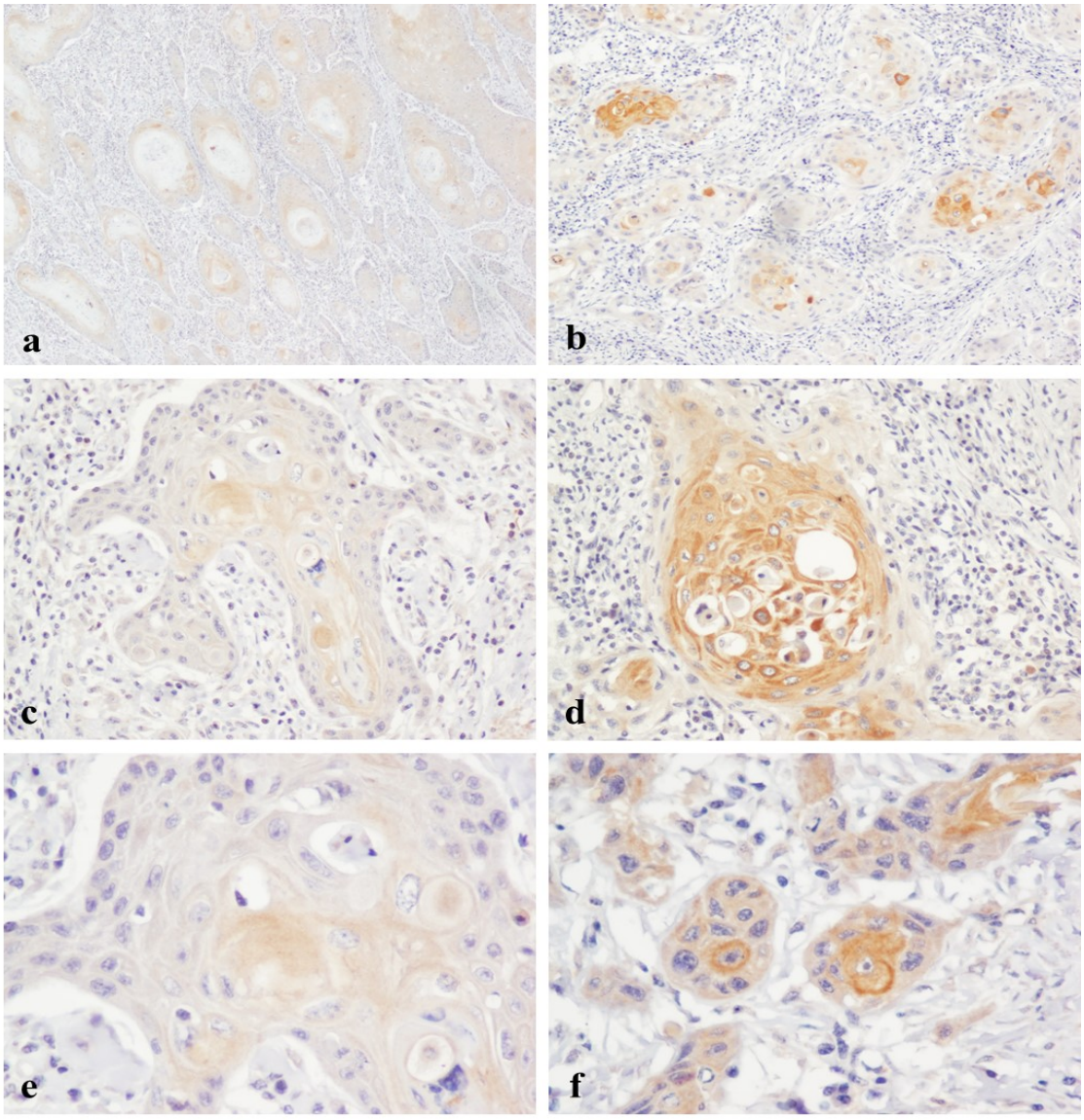

Fig 3.9 Immunoreactivity of cytokeratin 13 in OaPV3 positive SCC. Low magnification of weak **(a)** and strong **(b)** signals detected in malignant squamous cells. High magnification of

weak **(c, e)** and strong **(d, f)** immunoreactivity observed in the suprabasal layers of keratin pearls.

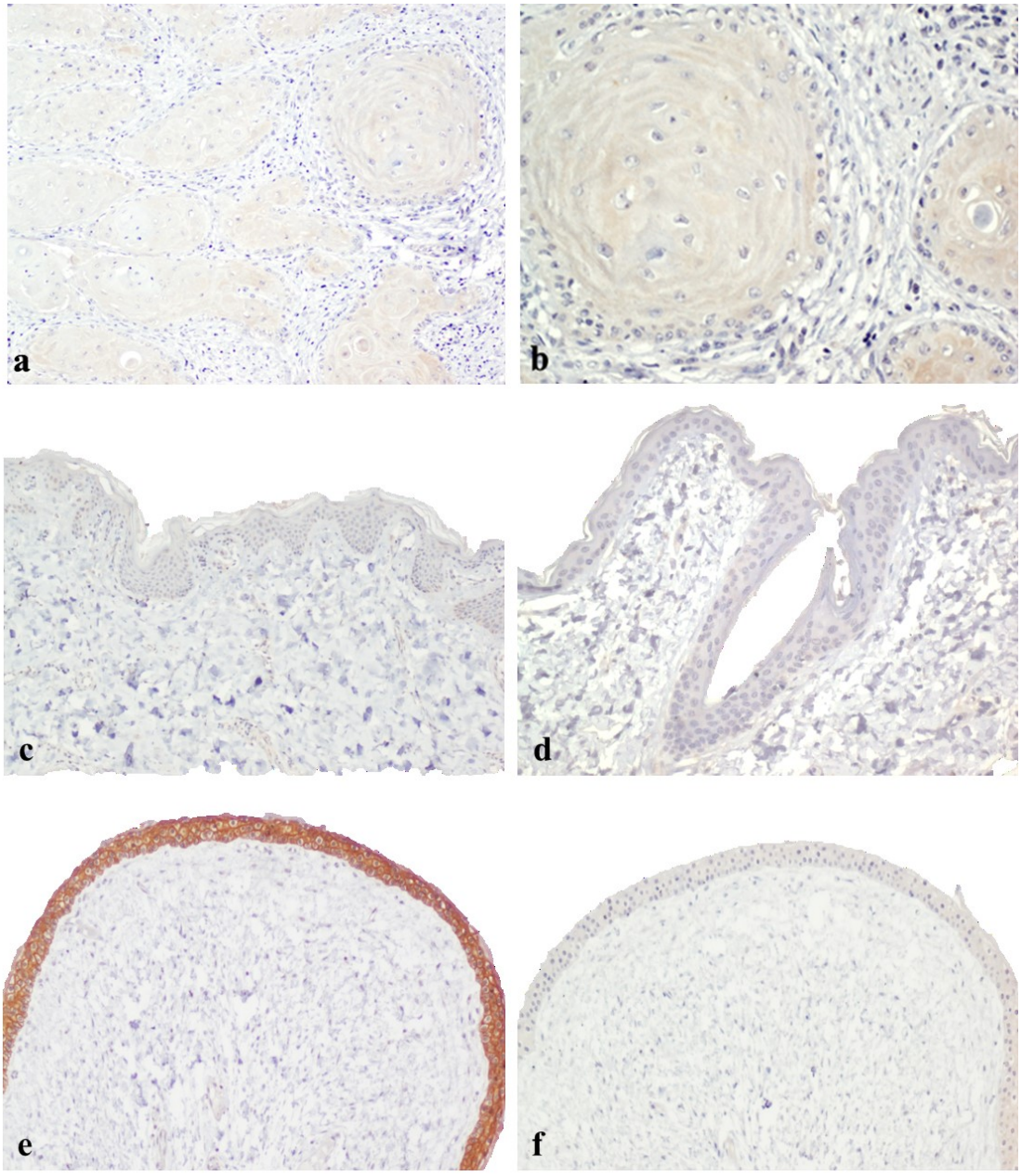

Fig 3.10 Immunohistochemistry of cytokeratin 13. Absence of immunoreactivity in **(a, b)** OaPV3 negative SCC and **(c, d)** non SCC samples. **(e)** Strong immunoreactivity signals detected in epithelial cells of urinary bladder used as positive control. **(f)** Absence of signal in the negative control.

Table 3.2 Immunohistochemical score for the cytokeratin 13.

Case No.	Site[a]	Histological evaluation	PCR results	IHC score
1	H	SCC	pos	pos
2	H	SCC	pos	pos
3	H	SCC	pos	pos
4	H	SCC	pos	pos
5	H	SCC	pos	pos
6	H	SCC	pos	pos
7	U	SCC	pos	pos
8	U	SCC	pos	pos
9	U	SCC	pos	pos
10	U	SCC	pos	pos
11	H	SCC	neg	neg
12	H	SCC	neg	pos
13	U	SCC	neg	pos
14	U	SCC	neg	neg
15	U	SCC	neg	neg
16	U	SCC	neg	neg
17	U	SCC	neg	neg
18	U	SCC	neg	neg
19	U	SCC	neg	neg
20	U	SCC	neg	neg
21	H	non SCC	neg	neg
22	H	non SCC	neg	neg
23	H	non SCC	neg	neg
24	H	non SCC	neg	neg
25	H	non SCC	neg	neg
26	H	non SCC	neg	neg
27	U	non SCC	neg	neg
28	U	non SCC	neg	neg
29	U	non SCC	neg	neg
30	U	non SCC	neg	neg

Abbreviations: H, head region; U, udder; PCR, polymerase chain reaction; IHC, immunohistochemistry; SCC, squamous cell carcinoma.

[a] Anatomic location of SCCs

3.4 Discussion

The study of protein expression levels involved in animals disease provide new insights in veterinary medicine, involving the molecular characterization of various pathological states and the ability to interpret the molecular mechanisms dictating the initiation and progression of the disease. The emerging proteomic approaches allows the identification of specific biomarkers that may be used as protein target for intervention and treatment of several disease (Lippolis and De Angelis, 2016). Based on these considerations, the proteomic analysis appears particularly interesting in cancer research, in which the study of tumor cell biology, as well as the identification of proteins involved in specific cellular process related to cancer, represent an important tool for tumors knowledge.

We analyzed different cutaneous samples, including non SCC samples and OaPV3 positive and negative SCCs, in order to identify differentially expressed proteins related to the OaPV3 infection.

Proteins related to stress and proteins involved in the negative regulation of apoptotic process and cell death, were the most represent in OaPV3 positive SCCs by the shotgun analysis. In literature, these changes are mostly associated with the pathological states in which cancer cells employ a number of different strategies to suppress a protective apoptotic response. Nevertheless, the proteins overexpression in OaPV3 positive SCCs compared to OaPV3 negative SCCs is indicative of an active viral role in the deregulation of processes related to homeostasis. In particular, apoptosis plays an essential role in the maintenance of tissue homeostasis and different signaling pathways, including survival and stress-induced signaling pathways, are able to directly modulate the apoptotic machinery, playing a central role of key regulatory molecules (Plati *et al.*, 2011). The apoptotic machinery can be broadly divided into two classes of components,

referred as sensors and effectors. The sensors are responsible for monitoring the extracellular and intracellular environment for conditions of normality or abnormality. In case of abnormal signals, including DNA damage, survival factor insufficiency, or hypoxia, the sensors activate the effectors which activate the apoptotic death. Many of the signals eliciting the apoptosis process converge on the mitochondria, which respond by releasing cytochrome C, a potent catalyst of apoptosis (Hanahan and Weinberg, 2000).

Abnormalities in cell death regulation can be a significant component of disease, including the tumorigenesis process. Cancer is considered the most important example in which the normal mechanisms of cell cycle regulation are dysfunctional, leading to an over proliferation and a decreased removal of cells (King and Cidlowski, 1998). The ability of cancer cells to evade the apoptosis process is considered an important hallmark of cancer (Hanahan and Weinberg, 2000). A variety of molecular mechanisms used by tumor cells to suppress apoptosis have been described, including the acquisition of resistance by the expression of anti-apoptotic proteins or by the down regulation of proapoptotic proteins. The expression of both family proteins is regulated by the p53 tumor suppressor gene, a transcription factor that regulates the cell cycle by the regulation at the G1/S point. Furthermore, p53 play an essential role in case of DNA damage by the activation of DNA repair process or the initiation of apoptosis process if the DNA damage proves to be irreparable (Elmore, 2007). Nevertheless, the p53 gene is frequently mutated in tumorigenesis process, leading to a reduction of tumor suppression. Papillomaviruses, as well as radiation and various chemical agents, can damage the p53 gene. In particular, the E6 protein of Papillomaviruses is able to specifically bind and inhibit the activity of the p53 product by two different mechanisms. A mechanism was firstly proposed by Scheffner and colleagues (1990)

that showed a specific bind between the E6 protein and p53 inducing a degradation of the p53 via the ubiquitin pathway. Another mechanism has been described by Crook and co-workers (1991) that proposed a specific interaction between E6 protein and p53, without degradation, that prevents the p53-mediated transcriptional repression of TATA-containing promoters. However, with or without degradation process, the repression of p53 increase the ratio of pro to antiapoptotic proteins, that negatively regulate the programmed cell death, promoting the tumorigenesis (Fridman and Lowe, 2003).

Based on these considerations, the increased protein levels of proteins involved in the negative regulation of apoptotic process and cell death detected in OaPV3 positive SCCs may be due to the viral infection, whose E6 protein could bind the p53 tumor suppressor protein inducing a deregulation of apoptosis process and an increment of antiapoptotic proteins levels. This hypothesis appears further confirmed by the 14-3-3 theta overexpression in OaPV3 positive SCCs. Although an overexpression of the 14-3-3 protein has been frequently observed in different human cancers, several studies showed the ability of the protein to directly interplay with the E6 viral protein (Jang *et al.*, 2004; Keshamouni *et al.*, 2006).

The 14-3-3 proteins comprise a large family of highly conserved, small, acid polypeptides of 28-33 kDa that are found in all eukaryotic species. The 14-3-3 proteins are specific phosphoserine/threonine-binding proteins and in humans, seven isoforms (β, γ, ε, ζ, η, σ and τ) have been identified. Most of the isoforms are expressed in all tissues, although 14-3-3 σ expression is restricted to epithelial cells (Hermeking, 2003). The targets of 14-3-3 include transcription factors, biosynthetic enzymes, cytoskeletal proteins, signaling molecules, apoptosis factors and tumor suppressors. The contribution of this protein in cancer has been reported in several studies, highlighting a common

mechanism of protein sequestration in the cell cytoplasm. The sequestration model was firstly proposed by Zha and co-workers (1996) after the identification of the 14-3-3 mediated cytoplasm sequestration of the proapoptotic protein BAD. The antiapoptotic effect of the 14-3-3 protein was further confirmed by Brunet and colleagues (1999) that demonstrated the cytoplasm sequestration of FKHRL1, inhibiting the ability of this transcription factor to stimulate apoptosis. Recently, Boon and Banks (2013) showed a specific interaction of the PDZ binding motif (PBM) localized in the extreme carboxy termini of the HPV E6 oncoprotein with the 14-3-3 protein. In particular, the PBM E6 protein of PV-inducing cancer seems to be involved in a wide variety of functions, including keratinocyte immortalization and transformation. In animal models, the integrity of the E6 PBM appears to play a role in the induction of malignancy in both skin and cervix. Furthermore, the phosphorylation of the E6 PBM confers the ability to interact with other potential cellular target proteins, including the 14-3-3 proteins and allowing an increased activity of their antiapoptotic effect (Boon and Banks, 2013). For all these reasons, the overexpression of 14-3-3 theta protein detected by the shotgun analysis may be due to the interactions with the OaPV3 E6 protein. Regrettably, our immunohistochemistry results did not confirm this hypothesis, probably because the polyclonal antibody raised against the 14-3-3 human protein is not able to detect the orthologous sheep protein, as confirmed by the diffuse staining pattern observed in dermal and epidermal structures. This process, referred as species cross-reactivity, results in a decreased antibody affinity in different species and, conversely, involves the antibody-unrelated epitopes binding (Ramos-Vara and Miller, 2013). Based on these considerations, further studies including an antibody panel for the 14-3-3 theta protein will be, thus, necessary to definitely confirm the overexpression of this protein in OaPV3 positive SCCs.

Results showing the increment of proteins related to stress and involved in the negative regulation of apoptotic process and cell death, reflect the significant down regulation of proteins involved in tissue development and epithelial cell differentiation, detected in OaPV3 positive SCCs. Proliferation and differentiation are tightly coupled processes that involve several cell cycle proteins, including the Rb and the cyclin family proteins. These proteins, also referred as transition proteins, are involved in the G1/S phase transition, allowing the cell cycle progression or, conversely, the cells terminal differentiation (Caldon *et al.*, 2010). Nevertheless, cell cycle proteins may be sequester or modify by others proteins, leading to an altered cell cycle progression. Interestingly, the E7 proteins of Papillomavirus is able to form complexes with the retinoblastoma tumor suppressor gene product, inducing its degradation and allowing the cell cycle progression (Munger *et al.*, 1989). For all these reasons, the down regulation of proteins involved in tissue development and epithelial cell differentiation seem to be further attested by the viral infection in SCCs, in which the PV may act several strategies to induce an altered cell cycle progression, inhibiting thus proteins involved in the regulation process.

To validate the biological relevance of proteomics findings, we looked for the altered expression of cytokeratin 13 detected in OaPV3 positive SCCs, a protein characteristic of mucosal stratified squamous epithelial cells and considered as a differentiation epithelial marker for non-keratinizing epithelium (Lam *et al.*, 1995). The cytokeratin 13 is normally expressed in the suprabasal layers of non-cornified stratified epithelia, particularly in esophagus, tonsil, and cervix (Malecha and Miettinen, 1991). The detection of its overexpression in OaPV3 positive SCCs appeared thus uncertain, considering also the down regulation of the others proteins involved in epithelial cell differentiation and tissue development, as galectin-7 and transgelin-2. Nevertheless, the

cytokeratin 13 presence is also reported in normal skin of human at low levels. Interestingly, Hudson and co-workers (2010) reported an overexpression of this protein in human cutaneous SCC. The findings of our study were thus novel, since no altered levels of this proteins were detected in OaPV3 negative SCC compared to non SCC samples, leading to consider the overexpression of this protein to be mostly related with the viral infection. Our hypothesis seems further attested by the immunohistochemistry results, confirming the high levels of this protein in examined OaPV3 positive SCCs. A barely immunoreactivity was also observed in two OaPV3 negative samples in which we cannot exclude the presence of load viral copies not detected by PCR.

Nevertheless, our data appeared conflicting to previous reports showing the ability of the HPV 16 E1^E4 to bind cytokeratin, inducing the collapse of the cell cytoskeletal network (Raj *et al.*, 2004). The cytokeratin 6 and 3 seem to be involved in this degradation process, showing down regulation levels in OaPV3 positive SCCs compared to OaPV3 negative SCCs. For this reason, our hypothesis is that the overexpression of cytokeratin 13 in OaPV3 positive SCCs may be related to a mechanism of compensation reported for others cytokeratin by Kanaji and co-workers (2006). In particular, type I and type II cytokeratins have been shown to form obligate 1:1 heteropolymers, suggesting that dynamic changes must occur in their expression levels, particularly when one cytokeratin is suppressed. The down regulation of cytokeratin 6 and 3 induce by the viral protein may activate a mechanism of compensation in which the cytokeratin 13 of the group I is expressed in order to compensate the absence of cytokeratin group II (cytokeratin 6 and 3). However, further studies will be necessary to validate this hypothesis.

3.5 Conclusion

Overall, to the best of our knowledge, this is the first proteomic approach investigating the deregulation of protein related to the viral infection in ovine tumors. The over or down regulated biological process seem directly involved in the viral pathogenesis, representing thus an important tool for the study of ovine SCC etiology. Although further and detailed analysis will be necessary in order to confirm our hypothesis, the cytokeratin 13 seems a promising biomarker candidate of the OaPV3 infection in ovine cutaneous SCCs.

The viral involvement in the etiology of cutaneous squamous cell carcinoma of sheep represents a new research perspective in which specific viruses-associated tumor may play a key role in cancer development. The results of this thesis, detecting OaPV3 and the related transcriptional activity in high number of samples, suggest that the virus can represent a driving force in neoplastic transformation, as proposed for several papillomavirus related tumor in human. The identification of altered molecular pathways involved in cell cycle and apoptosis, frequently reported in literature as alteration related to the viral activity, attested a specific virus-host interaction in which OaPV3 employs several strategies to induce malignant transformation. The discovery of viral related biomarkers, representing the goal standard of proteomic approach, appears particularly important for future studies in which the application of these proteins as diagnostic biomarkers of viral infection will be evaluated.

Although this thesis is a preliminary investigation and represent the first proteomic approach investigating the viral involvement in tumor of sheep, the OaPV3 infection seems to play a central role in cutaneous squamous cell carcinoma and, as proposed in

bovine species, the ovine can represents a model for comparative studies of viruses related to cutaneous tumors in human.

3.6 References

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"There are two primary choices in life:

to accept conditions as they exist,

or accept the responsibility for changing them."

Dr. Denise Waitely

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