An Investigation of Double Strand DNA Repair in Human Papillomavirus Mediated Gynaecological Cancers

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by

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Abstract

Background The DNA damage repair response is a key determinant of sensitivity to DNA damaging agents which are utilised for the treatment of gynaecological cancers. A significant proportion of cervical and vulval tumours are mediated by persistent high-risk human papillomavirus (HPV) infection, which is associated with superior clinical outcomes when compared to HPV negative disease. The aims of this study were to investigate key effectors of the repair of DNA double strand breaks in cervical and vulval cancer to elucidate a potential mechanism by which HPV status influences DNA repair proficiency. The study also sought to establish an in vitro model of gynaecological cancers using fresh tissue from cervical and vulval tumours that was capable of facilitating investigation of the kinetics of DNA DSB repair in these tumours.

Methods Immunohistochemical staining for phosphorylated ATM (pATM) and FANCD2 were performed across a tissue microarray of cervical and vulval tumours, with comparative evaluation on an oropharyngeal cancer array. RNA was extracted from fresh and FFPE cervical and vulval cancer tissue to perform transcriptomic profiling of cervical and vulval cancers via Nanostring technology, with a focus on DNA repair genes as part of the PanCancer codeset. Tissues were assessed for high-risk HPV presence via p16 status, DNA and RNA in-situ hybridisation and/or HPV 16/18 real-time qPCR. Primary cultures were established from fresh tissue collected and pilot studies performed to assess foci formation of RAD51, ATM and γH2AX at serial timepoints following exposure to ionising radiation. RAD51 immunofluorescence was also trialled in FFPE tissue.

Results Lower levels of pATM are found in a proportion of cervical and vulval tumours compared to adjacent normal tissue, which could be hypothesised to confer increased radiosensitivity particularly as low pATM was found to be an independent predictor of improved survival following chemoradiation in the comparator OPSCC group. HPV positive tumours have increased nuclear pATM staining compared to HPV negative tumours, and therefore low levels of pATM activation are not a mechanism for the increased radiosensitivity of HPV positive tumours.

The novel transcriptomic data comparing squamous cervical and vulval cancers highlighted common features of all tumours including increased expression of cell cycle genes and DNA repair genes, particularly of the homologous recombination repair pathway, with a trend towards increased expression of DNA repair proteins in HPV positive tumours. This analysis identified that gynaecological squamous cancers demonstrate low NR4A1/Nur77 expression, which could be a potential biomarker of resistance to treatment and merits further investigation. This work also demonstrated the significantly dysregulated cytokine profile of vulval tumours which may inform future immunotherapy studies and identified the Hedgehog pathway as a potential therapeutic target in HPV negative vulval cancer.

Primary cell cultures were successfully developed and characterised from fresh cervical and vulval tissue and pilot studies demonstrated the potential utility in assessing the dynamics of DSB DNA repair following ionising radiation.

Conclusion There is no clear relationship between HPV and reduced levels of transcription/expression in a comprehensive panel of double-strand DNA repair genes. This suggests that HPV does not alter the availability of cellular DNA repair factors, but perhaps alters the proficiency of DNA damage repair by other mechanisms which can be studied further in the developed primary cell resource.

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List of Abbreviations

ATCC	American Type Culture Collection
ATM	Ataxia-telangiectasia-mutated
ATR	Ataxia-telangiectasia and RAD3-related
cDNA	Complementary deoxyribonucleic acid
CIN	Cervical intraepithelial neoplasia
DAB	3, 3'- Diaminobenzidine
DAPI	4', 6 – diamidino-2-phenylindole
DDR	DNA damage response
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DNA DSB	DNA double-strand break
DNA SSB	DNA single-strand break
DNA-PK	DNA-dependent protein kinase
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ETOH	Ethyl alcohol
FA	Fanconi anaemia
FAAP24	FA core complex associated protein
FFPE	Formalin fixed paraffin embedded
GMEM	Glasgow minimal essential medium
H&E	Hematoxylin and eosin
HPV	Human papillomavirus
HR	Hazard ratio

HRD	Homologous recombination deficiency
HR-HPV	High risk human papillomavirus
HRR	Homologous recombination repair
ICL	Interstrand crosslink
IF	Immunofluorescence
IR	Ionising radiation
ISH	In situ hybridisation
miRNA	Micro ribonucleic acid
mRNA	Messenger ribonucleic acid
NHEJ	Non homologous end joining
NOK	Normal oral keratinocytes
OPSCC	Oropharyngeal squamous cell cancer
OS	Overall survival
PARP	Poly-ADP ribose polymerase
pATM	Phosphorylated Ataxia-telangiectasia-mutated
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
PFS	Progression free survival
PI3K	Phosphoinositide 3-kinase
RNA	Ribonucleic
RPA	Replication protein A
TCGA	The cancer genome atlas
TERT	Telomerase reverse transcriptase
TMA	Tissue microarray
UV	Ultra violet
VAIN	Vaginal intraepithelial neoplasia
VIN	Vulval intraepithelial neoplasia

Chapter 1. Introduction

1.1 Human Papillomavirus-associated Gynaecological Cancers and Current Treatment Strategies

High risk strains of the Human Papilloma Virus (HR-HPV) are widely prevalent and implicated in the aetiology of cervical cancers, vulval cancers and vaginal cancers (99%, 43% and 70% respectively) (1). The predominant strains implicated are HPV 16, 18, 31 and 33, which are widely transmitted via direct contact with infected epithelium (2). Together these gynaecological tumours present a significant global health burden, with cervical cancer ranking as the 4th most common cause of cancer related death in women worldwide with approximately 300,000 deaths per annum (3). The identification of HPV as an aetiological agent of cancer has led to the development of HPV vaccination and HPV screening programmes in many nations, which is anticipated to significantly reduce the incidence of HPV-mediated cancers over coming decades (4). There remains a large population of women at risk from HPV-related gynaecological malignancies who have not been vaccinated and whilst the World Health Organisation strategy towards elimination will be a breakthrough in the prevention of these diseases over coming decades, there remains a need to improve treatments for patients who develop these diseases, even if the elimination target of <4 cases per 100,000 women-years is met in many years to come.

The therapeutic modality for early stage disease in vulval, vaginal and cervical cancers is surgery, with or without adjuvant radiotherapy. Locally advanced disease is treated with ionising radiation which is often delivered with platinum chemotherapy (cisplatin), which acts as a radiosensitiser. Unfortunately, many patients have residual disease or disease recurrence following chemoradiation and in locally advanced cervical cancer the overall 5 year survival ranges from 79% to 27% depending on stage of disease (5). Therapeutic options for women with recurrent, persistent, or metastatic cervical, vulval and vaginal cancer are limited. Response rates for women receiving platinum-containing doublet chemotherapy for recurrent, persistent, and metastatic regimens are between 22-29% (6). Some progress has been made with the addition of the anti-angiogenic agent bevacizumab in this setting, which increases the median overall survival by 3.7 months versus chemotherapy alone (7). More recently the KEYNOTE-826 trial demonstrated a benefit in adding pembrolizumab immunotherapy to chemotherapy +/- bevacizumab treatment in the first line setting, with improved median progression-free survival of 2.2 months and improved overall survival at 24 months (HR 0.64) (8). It is anticipated that this data will

soon result in immunotherapy being incorporated into first-line treatment schedules for cervical cancer patients in the UK. Despite these advances, there is very limited scope for second line treatment in the metastatic, recurrent, and persistent cervical cancer setting, as reflected by the poor survival outcomes in this setting. Currently only chemotherapy treatments are approved for metastatic, recurrent, or persistent vulval cancers in the second line setting, again with modest survival outcomes (9).

Targeted therapy with PARP inhibition has been widely incorporated into the management of another type of gynaecological malignancy (ovarian cancers), and these agents are an attractive strategy in tumours sensitive to DNA damaging agents, most strikingly in the case of high grade serous ovarian cancers in patients with inherited defects in DNA DSB repair (10). Pre-clinical data in cervical cell lines and xenografts demonstrates that PARP inhibition can suppress the growth of multiple cervical cell lines, demonstrating delayed DNA repair kinetics and increased sensitivity to concurrent DNA damaging agents (11-13).

Given the reliance on DNA damaging agents to treat this group of tumours, and the promising pre-clinical data with PARP inhibitors, a greater understanding of the activity of DNA Damage Repair (DDR) may lead to novel therapeutic approaches to further sensitise tumours to treatment and improve outcomes.

DNA damaging therapies such as Chemotherapy and ionising radiation (IR) activate the DDR via the induction of multiple DNA lesions, including DNA single-strand breaks (SSB), inter-strand crosslinks (ICLs) and DNA double strand breaks (DSB). DNA DSB are the most genotoxic and can arise directly as a result of IR or indirectly from other types of damage, particularly when DNA lesions such as ICLs encounter a DNA replication fork in S-phase. In the context of overwhelming DNA damage in replicating cells, the DDR activates programmed cell death (14).

Cancer cell survival upon exposure to these treatments depends on the cells' ability to withstand and repair DNA DSB and avoid apoptosis. It is recognised that HPV positive tumours are more sensitive to radiation therapy than HPV negative tumours arising from the same anatomical location (15-17) and it is therefore hypothesised that HR-HPV mediated carcinogenesis may impair the tumour's ability to withstand DNA damage, but a potential mechanism for this has not yet been elucidated.

1.2 The DNA Damage Repair Pathway

A background level of DNA damage occurs in all cells due endogenous and exogenous factors such as reactive oxygen species and UV radiation. The DNA damage repair response (DDR) exists to respond to DNA damage by halting cell cycle progression to facilitate the repair of DNA lesions to prevent mutagenesis and genomic instability. This process is an important mechanism to avoid carcinogeneiss in normal tissues, and if defective can lead to an increased incidence of malignancy (18).

There are two major pathways by which DNA DSB are repaired in mammalian cells: homologous recombination repair (HRR) and non-homologous end joining (NHEJ). HRR utilises the replicated sister chromatid as a template to accurately repair the lesion, and therefore is the method of choice during late S/G2 phase of the cell cycle. HRR activity is crucial to prevent mutagenic DNA changes from persisting in cells following replication (19). NHEJ directly joins the broken ends of DNA with minimal processing and is therefore more error-prone and is consequently suppressed in S/G2 in favour of HRR. The type of dsDNA repair carried out at each site of damage largely depends upon which point in the cell cycle the damage is sustained, and which proteins predominantly bind to the broken ends of DNA. Several key HRR proteins are selectively expressed or activated by phosphorylation during S/G2 (20, 21).

1.2.1 Homologous Recombination Repair (HRR)

For HRR to take place the DNA DSB is bound by the MRN complex (MRE11, RAD50, NBS1) (Figure 1.1) activating end processing of the 3' end of dsDNA by MRE11, exonuclease 1 and CtIP (22). ATM PI-3 kinase is recruited to the site of DNA DSB and is activated by autophosphorylation at serine 1981. Phosphorylated ATM (pATM) directly and indirectly (via activation of Chk 2) phosphorylates proteins including γH2AX and BRCA1 triggering the activation and recruitment of proteins required for HRR (23).

End processing by the MRN complex exposes ssDNA which is bound by replication protein A (RPA), and BRCA1 (associated with BARD1), BRCA2 (associated with PALB2), BRIP1, Fanconi Anaemia proteins (FANCD2, FANCG) and XRCC3 (24) are recruited to the site of DNA DSB. These proteins facilitate the loading of RAD51 recombinase onto the 3' overhang of DNA which displaces RPA and forms a nucleoprotein filament structure which can search for sequence homology with the sister chromatid to identify the correct template for DNA repair (25). Once such a sequence is found RAD51 facilitates strand invasion and utilises the

new DNA association to create a new section of DNA based on the sister chromatid template. The product of this is a Holliday Junction, which is resolved to complete the process of DNA repair and leave behind a fully intact sister chromatid (26).

1.2.2 Non Homologous End Joining (NHEJ)

In contrast to HRR, NHEJ can occur at any point during the cell cycle, although primarily utilised in G1 when Homologous recombination repair is not possible and proteins promoting NHEJ preferentially bind to the DNA site (27). It may also occur preferentially if

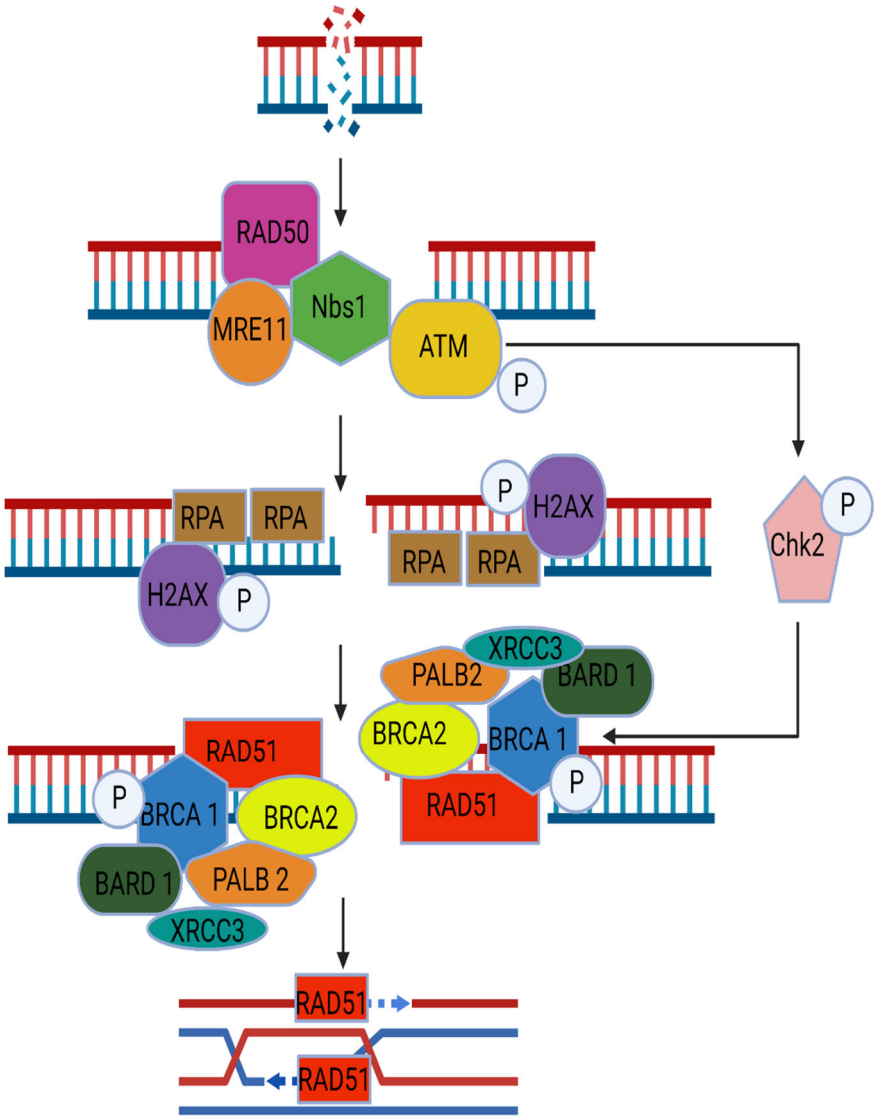


Figure 1.1 Homologous Recombination Repair (HRR) of Double Strand DNA Breaks

HRR is absent or compromised. For NHEJ to take place the DNA DSB is protected from end resection by binding of the Ku70/Ku80 heterodimer, building a synaptic complex including Ku70/Ku80, 53BP1, MDC1 and E3 ubiquitin ligase RNF8/RNF168 (28). Ku70/Ku80 then recruits DNA dependent protein kinase (DNA-PK) which is auto-phosphorylated and regulates the process of aligning the two ends of DNA. Ligase 4 complexed with XRCC4 and interacting with XLF then join the two ends (29). If there has been damage to bases flanking the site of DNA break, then other processing by Artemis nuclease can occur but this is error-prone due to lack of DNA template (30).

1.2.3 The Fanconi Anaemia Pathway

A specific type of DNA lesion induced by platinum chemotherapy is the inter-strand cross link (ICL) where two nucleotides are covalently linked together, preventing DNA strand separation for replication. If unresolved the stalled replication fork can collapse forming a DNA DSB, which requires repair by HRR. A part of the DDR named the Fanconi Anaemia (FA) pathway is thought to be important in resolving ICLs at stalled converging replication forks, preventing DNA DSBs. FA pathway is made up of 22 proteins (FANC A-W) and shares proteins with the HRR pathway including BRIP1/FANCJ, BRCA1/FANCS, RAD51/FANCR, PALB2/FANCN and BRCA2/FANCD1.

ATR PI3 kinase is activated by RPA and TopBP1 at sites of ICL (Figure 1.2) and directly, and indirectly by activating Chk1, it phosphorylates FA proteins including FANCM which identifies branched DNA at a stalled replication forks in association with FAAP24 (FA core complex association protein 24), MHF1/FAAP16 and MHF2/FAAP10 (31) . This provides a platform for the assembly of the rest of the FA core complex at the site of the ICL including FANC A/B/C/E/F/G/L/T/FAAP100/FAAP20 (32). BRCA1 also plays a role on clearing the site of ICL to allow core complex formation (33).

Core complex formation results in the mono-ubiquitination of the FANCD2/FANCI heterodimer by the E3 ligase activity of FANCL and E2 ubiquitin-conjugating enzyme FANCT/UBE2T, resulting in retention of FANCD2/FANCI on chromatin at the site of ICL (31, 34, 35). The efficiency of FANCD2 mono-ubiquitination is influenced by the ubiquitinated form of PCNA (36). Regulation of the recruitment of FANCD2/FANCI to sites of damage is by ATM/Chk2 and ATR/Chk1 mediated phosphorylation of these proteins (37-39).

The mono-ubiquitinated FANCD2/FANCI heterodimer acts with other FA proteins (FANCP/SLX4, FANCQ/ERCC4/XPF – ERCC1 heterodimer) and multiple nucleases (FAN1,

MUS81/EME1) to create nucleolytic incisions flanking the ICL on one strand of DNA, releasing it from the ICL (40). FANCD2/I heterodimer promotes translesion synthesis (TLS) to complete DNA synthesis of the intact strand which then forms a template for the repair of the DNA DSB in the sister chromatid by HRR, in which FA proteins such as FANCD2 and FANCG may also play a role (41).

1.2.4 Activation of Cell Cycle Arrest and Apoptosis

To facilitate the repair of DNA lesions encountered in the replicating cell, a reversible cell cycle arrest is coordinated by the DDR until such a time as the DNA is repaired. If DNA damage is encountered such that it overwhelms the cellular repair mechanisms, then apoptosis is triggered to prevent the survival and replication of cells with high levels of damage/mutations. Genes involved in this process act as tumour suppressor genes. The efficacy of DNA-damaging therapeutics is dependent on the ability of the target cell to repair DNA damage and to activate cell death in the event of catastrophic levels of DNA damage.

A key regulator of these processes is the transcription factor p53, whose levels are regulated by ubiquitination by Mdm2 E3 ubiquitin ligase which activates proteasomal degradation of p53 (42). In response to DNA damage or cellular stress, Mdm2 is phosphorylated by ATM and p53 is phosphorylated by ATM/ATR/Chk2/Chk 1 and DNA PK, inhibiting mdm2 mediated ubiquitination of p53, suppressing its degradation and activating the transcriptional activity of p53 (Figure 1.3). It is also likely that other factors influence transcriptional activity of p53 such as acetylation, p53 gene expression, p53 mRNA stability, and interactions between Pirh 2-p53 and ARF-Mdm2 (43, 44).

The fate of cells upon activation of p53 is thought to depend on the extent of p53 activation, but also the dynamic changes of p53 over time (45). Pulsed, short-lived activation of p53 is thought to be more likely to lead to a reversible cell cycle arrest for DNA repair, whilst sustained damage is more likely to result in cellular senescence and limitation of the growth of damaged cells. High levels of p53 activation beyond a certain threshold promote apoptosis, possibly due to higher affinity p53 binding sites on cell cycle arrest gene promoters than on pro-apoptotic gene promoters (46). Thus, the determination of cell fate is a complex process, influenced by post-translational modifications of p53, and likely related to relative levels of key cell cycle arrest/pro-apoptotic proteins (45, 47).

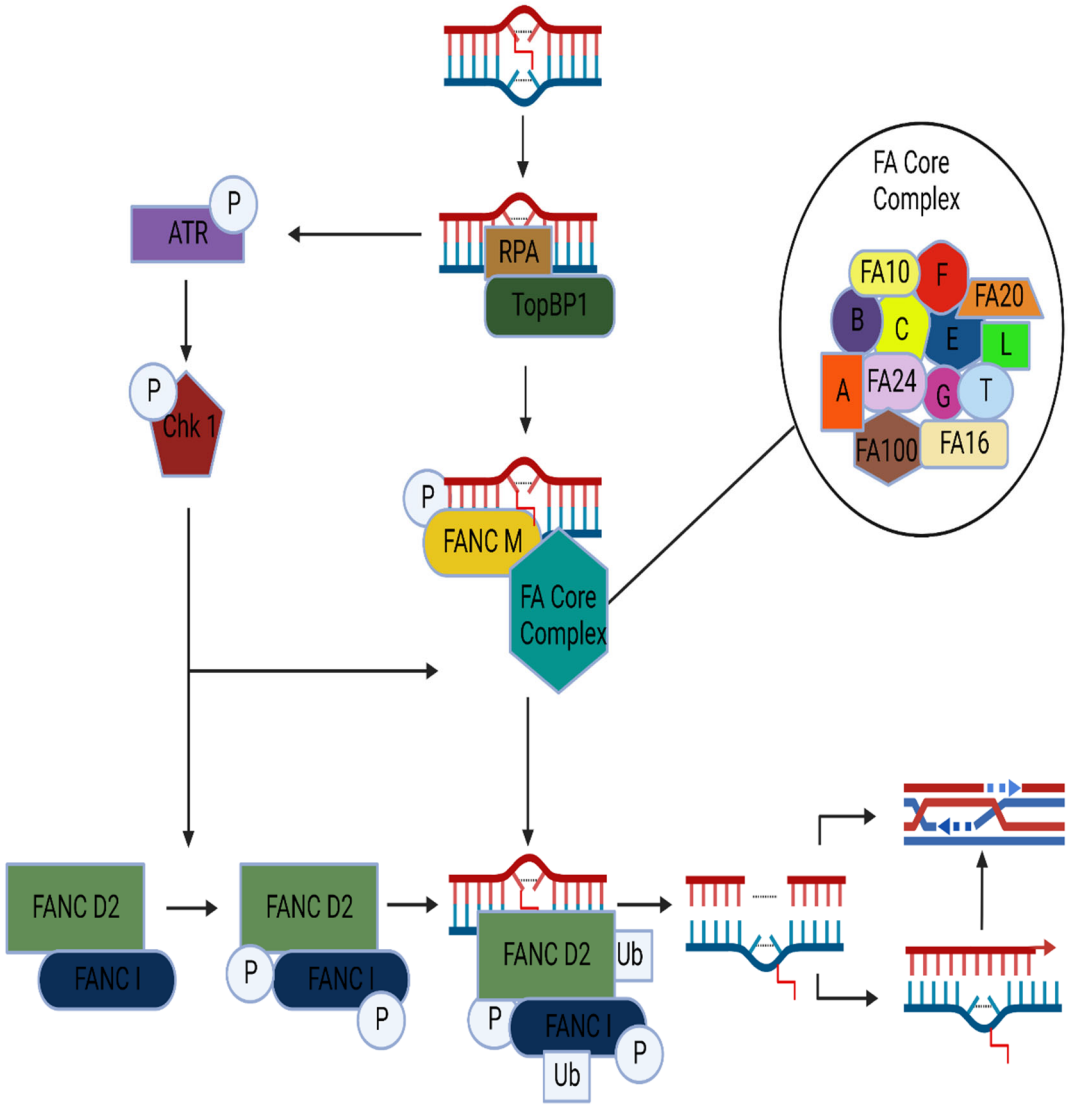


Figure 1.2 – Inter-strand Crosslink Repair by the Fanconi Anaemia Pathway.

A gene highly responsive to p53 induction is the cyclin-dependent kinase inhibitor p21 which facilitates cell cycle arrest at multiple junctures by inhibition of cdk2/cyclin E, cdk4/cdk6/cyclin D, cdk1/cyclin B, and cdk1/cyclin A (48). This in turn prevents phosphorylation of retinoblastoma protein (Rb), which remains in a bound state to the E2F transcription factor, repressing the transcription of genes required for cell cycle progression and DNA replication. p21 also inhibits DNA replication by direct interaction with PCNA and DNA Polymerase δ (48).

High levels of p53 activation induce pro-apoptotic genes including BH3 domain-only proapoptotic proteins which activate the intrinsic apoptosis pathway via mitochondrial outer membrane permeabilization (Puma, Noxa, Bad, Bax, Bak, p53AIP1) and death receptors/apoptotic execution factors which facilitate the extrinsic apoptosis pathway (Fas, Dr4, Killer/Dr5, Apaf1, caspase 6, Bnip3L) (47). There is also evidence that p53 directly interacts with pro-apoptotic factors in mitochondria to promote apoptosis (47).

In summary, tumour response to DNA damage is dependent on a complex interplay between multiple cellular pathways and appropriate recruitment and activation of their components. It is recognised that disruption in these pathways (such as p53 mutations, ATM mutations, BRCA mutations) not only predispose to cancer development but can influence the response to DNA damaging therapies (18). Persistent HPV infection promotes the development of dysplasia and cancer through mechanisms which alter cellular response to stress, including DNA damage, to tip the balance in favour on ongoing proliferation versus cell cycle arrest and allow acquired mutations to be passed on to progeny cells.

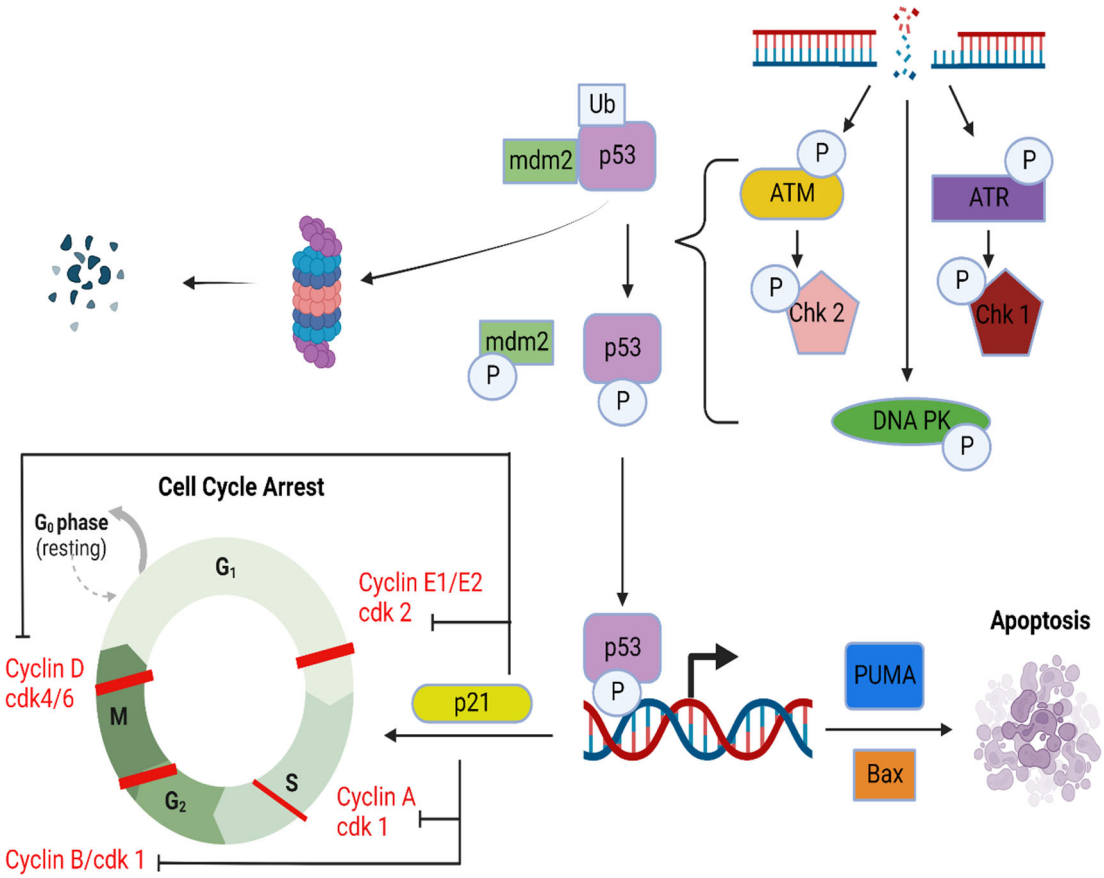


Figure 1.3 p53 activation of Cell Cycle Arrest and Apoptosis.

1.3 The HPV Life Cycle

The human papillomaviruses are DNA viruses from a large family containing over 200 types, about 40 of which can infect the genital areas and of these 15 (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) are termed high risk (HR-HPV) and having oncogenic potential. Low risk types often cause no disease but can cause genital warts.

The HPV genome is shown in Figure 1.4 and consists of early (E) and 2 late (L) genes. E1 and E2 are necessary for viral replication as they recognise the viral origin of replication. E1 acts as a helicase to facilitate DNA replication and E2 acts as a transcriptional regulator of viral genes (49). The E4 gene contributes towards genome amplification, binds to cytokeratin structure of host cells and is implicated in virion release (50)

E5, E6 and E7 are considered viral oncogenes (Figure 1.5). E6 mediates the ubiquitination and degradation of p53 by facilitating interaction between E6-AP (an E3 ubiquitin ligase) and p53 (51, 52). The E7 protein has a high affinity for binding Rb protein and its family members p107 and p130, resulting in the release and activation of E2F transcription factors. Therefore, both E6 and E7 contribute to loss of cell cycle G1 arrest in response to DNA damage and contribute towards cell transformation (53, 54). E5 is also implicated in this process but the mechanism of this is less well defined, and possibly due to interaction with growth factor receptor signalling (55).

The late genes, L1 and L2 are capsid proteins expressed in the later phases of HPV infection when virions are released from the epithelial surface for transmission.

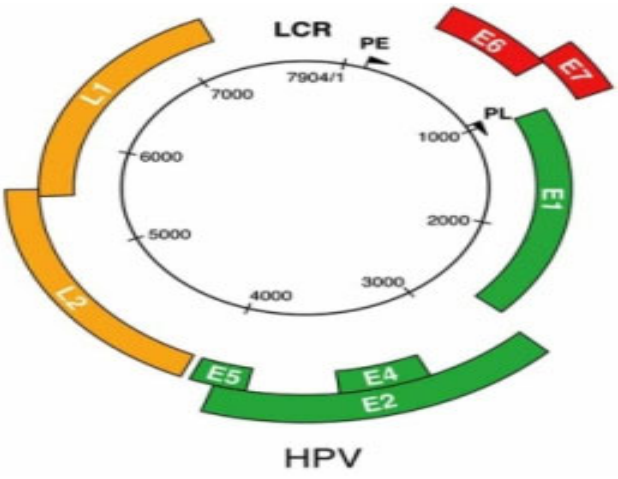


Figure 1.4 The HPV genome adapted from Cid-Arregui et al 2009[56]

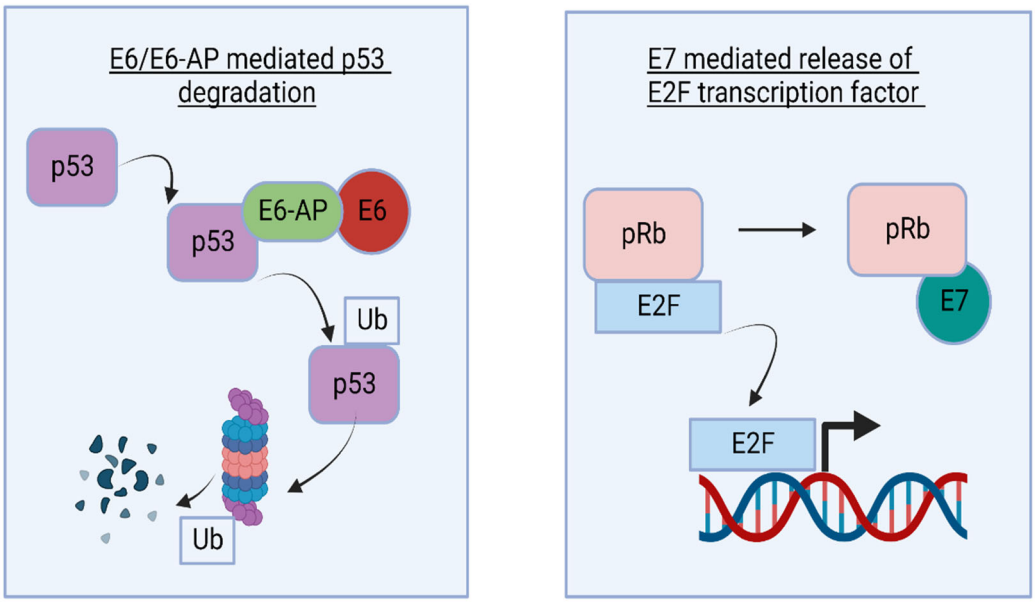


Figure 1.5 p53 and E2F dysregulation by HR-HPV E6 and E7

1.3.1. HR-HPV Infection and Maintenance Phase in Basal Epithelium

The HPV virus enters the basal layer of the squamous epithelium in areas where there is loss of epithelial integrity. HPV genomes exist in extrachromosomal, nuclear, episomal form and HPV copy numbers are retained by replication of episomal DNA during S phase alongside cellular DNA replication, as the HPV genome does not include a DNA polymerase (Figure 1.5). During this maintenance phase, the expression of early viral proteins E1 and E2, which target cellular replication proteins to the viral origin of replication, are supressed by host miRNA 145 and E1, the viral helicase, is exported into the cytoplasm by Crm1 dependent export (57, 58). This supresses viral genome replication to low levels.

1.3.2 HR- HPV infection in Differentiated Keratinocytes (Amplification Phase)

When infected keratinocytes begin to differentiate in the upper layers of the epithelium, they divide more rapidly and the amplification phase of the HPV life cycle initiates, with an elevation of E1 and E2 levels and a retention of E1 in the nucleus (Figure 1.5). HPV E2 protein in complex with BRD4 promotes tethering of the viral genome to cellular DNA at sites of replicative stress and forms a viral replication centre with E1 and the viral episome

(59). This ensures a good supply of replicative factors and that viral genomes will be divided between daughter cells on mitosis alongside genomic DNA. Viral replication centres induce a localised DDR activation which is necessary to provide the cellular machinery required for viral DNA replication (51, 60), which may occur outside of S phase due to the presence of E1/E2 which are not inhibited by the cellular checkpoint response. Many of the proteins involved in DNA DSB repair have been identified as localizing at centres of viral replication, in particular those associated with HRR, and it is possible that during genome amplification HPV uses a recombination-dependent mechanism of DNA replication (61).

To prevent constitutive activation of the DDR from abrogating cell cycle progression and inducing apoptosis, HPV oncoproteins E6 and E7 inhibit p53 and Rb respectively (Figure 1.5). There is also evidence that E7 increases the levels of SIRT1, which has been identified as a negative regulator of p53 (62).

1.4 HPV associated Oncogenesis

Most women will encounter a HR-HPV infection (HPV 16, 18, 31 and 33) at some stage in their lives, but this rarely causes the development of an HPV-associated high grade dysplasia or cancer. This largely reflects the ability of the host immune system to clear the virus. In cases where HR-HPV viral persistence occurs, there is a well-recognised spectrum of intraepithelial neoplasia (CIN, VIN, VAIN) which at an early stage most commonly regresses but can lead to the development of invasive cancer if left untreated (Figure 1.5) (63). The development of such malignancies is largely attributed to the oncogenic effects of high risk E6 and E7 proteins which have been associated with chromosomal instability and double-stranded DNA breaks in infected keratinocytes (64, 65). However, the presence of high risk E6 and E7 is not sufficient to directly cause the development of cancer. It is likely that chromosomal instability causes cells to develop oncogenic mutations during a persistent infection with high levels of E6/E7 expression which are passed onto daughter cells in the context of aberrant cell cycle control and inappropriate DNA damage checkpoint recovery (53, 66).

Integration of the episomal HPV genome into the cellular genome is also recognised as a key event in the development of HPV-associated cancers (67). As HPV does not encode an integrase gene, this process is likely facilitated by genomic instability and DNA DSB, with integration more commonly occurring at common fragile sites (68-72). There is some evidence for viral insertion in proximity to oncogenes and altered transcription of these

following HPV genome insertion (73), however this does not appear consistently and is unlikely to play a major role in the development of HPV associated cancers (70, 71, 74, 75).

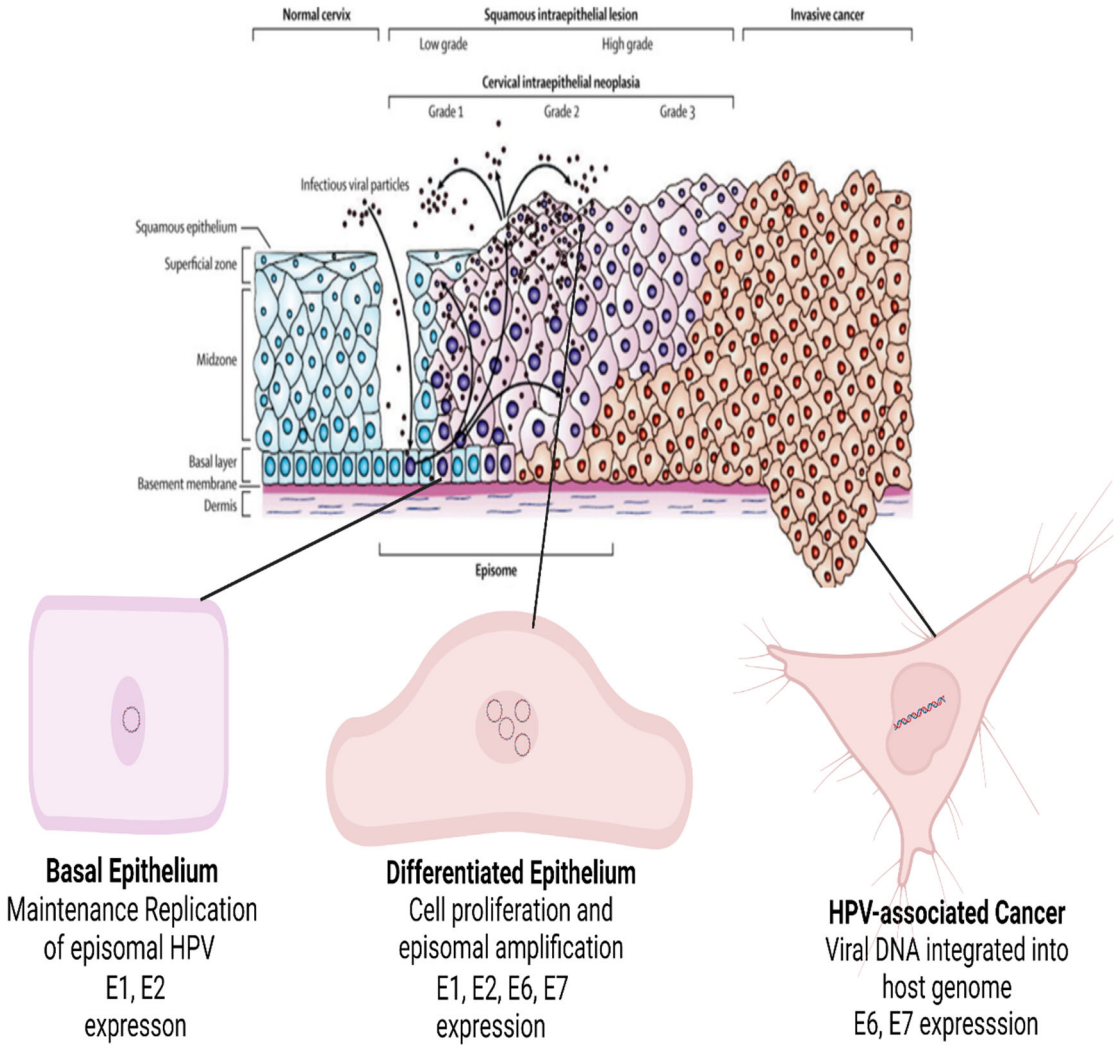


Figure 1.6 HR-HPV carcinogenesis in Squamous Epithelium adapted from Cohen et al (76)

It is more likely that HPV genome integration contributes to the oncogenic process by promoting E6/E7 expression in the basal layers of the epithelium, in contrast to the usual site of E6/E7 expression in differentiated keratinocytes in the upper layers of the epithelium, which are shed during the normal process of skin maintenance. During HPV genome integration, there is often evidence of loss of E2, the transcriptional regulator of the E6/E7 promoter region (36)(77). There is also evidence that viral genomic integration may lead to increased mRNA stability of E6 and E7 transcripts (78). Both of these circumstances may increase E6/E7 oncoprotein levels and promote unregulated cellular proliferation (78-80).

E6 and E7 have other putative oncogenic roles in addition to their effects mediated by p53 and Rb. E6 has been identified as promoting hTERT expression which may contribute to cell survival by maintaining telomere length (81). E7 has also been implicated in the acceleration of claspin degradation, resulting in DNA damage checkpoint recovery in G2 which is permissive of cellular division in the presence of unrepaired DNA damage (82-84).

1.5 The Relationship between HR-HPV infection and DNA Damage Repair in Gynaecological Cancers

As highlighted thus far in this chapter, HR-HPV infection can significantly alter the cellular response to stress and DNA damage, but the extent to which DDR pathways are altered in HPV-associated gynaecological malignancies, and how this may influence treatment outcomes, is yet to be fully established (61).

1.5.1 Fanconi Anaemia Syndrome is Associated with an increased risk of HPV-associated Gynaecological Cancers

The rare clinical syndrome, Fanconi Anaemia (FA), is a recessive genetic disorder associated with loss of any one of the 22 proteins which make up the Fanconi Anaemia family, with resulting loss of functionality of the FA pathway and chromosomal instability. FA patients have a general predisposition toward the development of haematological and solid malignancies, contributed to by the loss of HRR function and increased NHEJ in S phase, a more error-prone mechanism of repair with increased potential for malignant transformation (85, 86). The observed frequency of anogenital squamous cancers significantly exceeds that of other solid malignancies in this group of patients and therefore it is possible that the loss of FA pathway activity is particularly deleterious in the context of HPV malignant transformation.

Two studies have identified HPV DNA associated with the majority of anogenital cancers in FA patients (87, 88), although this finding is not universal (89). The apparent absence of HPV in some of these tumours may be due to the accumulation of sufficient chromosomal damage that ongoing E6/E7 expression is no longer needed for tumour propagation, which may account for the absence of detection of HPV in some of these tumours (90).

Possible mechanisms for this high incidence of gynaecological squamous cancers in the FA population include enhanced viral persistence (91), increased propensity for viral genomic integration at common fragile sites (92) and increased oncogenic potential of HR-HPV oncogenes(93).

In vitro studies and FA deficient transgenic mice models demonstrate an important role for the FA pathway in supressing E7 mediated oncogenesis (94-96), showing that loss of FA pathway activity in HPV positive immortalised keratinocytes leads to increased cellular and viral replication, increased levels of E7, and increased chromosomal abnormalities. Similarly, FANCD2 deficient cells can be transformed by the transfection of HR-HPV E6 or E7 alone (97). These associations have led to interest in evaluating the activation status of the FA pathway in HPV-associated cancers arising in patients the non-FA population. One hypothesis is that a degree of loss of functionality of the FA pathway may predispose individuals to HPV mediated oncogenesis at these sites. A second hypothesis is that HPV infection may somehow impair the functionality of the FA pathway, and that this may be a step towards malignant transformation in HPV-associated cancers.

There is little conclusive data from polymorphism studies to define the role of FA polymorphisms/SNPs in predisposition to squamous gynaecological cancer. Two large studies, in Sweden and Costa Rica, assessed FA protein polymorphisms in populations of women with CIN 3/invasive cancer against matched controls. The Costa Rican study (91) demonstrated that the *FANCA* G501S polymorphism was associated with an increased risk of CIN 3/cancer. This however was not replicated in the findings from the Swedish population (98). There have been smaller studies that supported an association between SNPs in *BRCA 2/FANCD1* and *FANCJ/BRIP 1* with an increased risk of cervical cancer (99, 100) and a study in the Chinese Han population, found that the SNP rs7213430 at the 3'UTR of *FANCJ* was associated with susceptibility to cervical cancer (100).

The Cancer Genome Atlas survey of cervical cancer identified a low level of mutations in FA pathway genes in cervical cancer specimens, the most prevalent of which are in *FANCA* (2.7%) and *FANCD2* (2.1%) (101) while whole Exome Sequencing of vulval cancer,

demonstrated a nonsense mutation in *FANCA* in 1 out of the 6 HPV positive specimens (102). These findings are in keeping with *FANCA* being recognised as the most frequently mutated FA gene across many different populations and as the gene most commonly responsible for the phenotype of FA syndrome if mutated copies are acquired from each parent (103). The carriage of mutated FA genes (*BRCA2/FANCD1, FANCC, FANCJ/BRIP1, FANCD2*) has been shown to increase the risk of certain cancers, predominantly breast and ovarian, and a Swedish population study demonstrated that carriers of mutations in *BRCA2/FANCD1* have an increased risk of cervical cancer (99). There also is evidence to suggest that *BRCA1/FANCS* mutation carriers have an increased relative risk of developing cervical cancer when compared to the general population (3.72 fold) (104). However, given the small numbers of FA gene mutations identified and the population prevalence of cervical cancer, it is unlikely that the carriage of FA gene mutation is a major pre-disposing factor to HPV-associated oncogenesis.

The acquisition of FA pathway impairment in tumours during the process of HPV-associated oncogenesis is a more likely hypothesis and pre-clinical studies suggest a role for the FA pathway in limiting the chromosomal instability caused by HPV E7 action. Therefore, acquired impairment of FA activity during HPV infection could potentially increase the risk of malignant transformation, but this hypothesis has not been extensively studied and there is little conclusive data regarding the status of FA pathway functionality in HPV-associated cancers and how this could potentially influence tumour therapeutic response.

Epigenetic analyses have shown that hypermethylation of the *FANCF* gene was found in 30% of primary cervical tumours studied with a significantly higher frequency in younger patients, although these changes were not observed in pre-malignant cervical smears (105). The same study also identified *FANCF* hypermethylation in 3 secondary cell lines, which demonstrated down-regulated expression of *FANCF*, and 3 other cell lines demonstrated down-regulated *FANCF* mRNA in the absence of promoter hypermethylation.

FANCD2 foci are increased in the presence of HR-HPV E7 in vitro, and UVIN samples demonstrate upregulation of *FANCD2* gene expression when compared to normal vulval epithelial/low risk HPV condylomas (84, 106), but the activation status of the FA pathway is unknown in pre-malignant and malignant lesions. A greater understanding of the functionality of the FA pathway in HPV mediated tumours is required to test the hypothesis of impaired functionality contributing to HPV carcinogenesis.

1.5.2 Homologous Recombination Repair in the presence of HR-HPV

HR-HPV oncoproteins E1 and E7 cause increased levels of DSB DNA breaks, activating the DDR via ATM and phosphorylation of downstream proteins (107-109). This is also seen in the presence of HPV 16, 18 and 31 genomes, although to a lesser degree as with ectopic E1 expression, possibly due to the co-activity of E2 (107, 109, 110). E6 mediated degradation of p53 has been shown to induce upregulation of BRCA1 and increase RAD51 activity (111, 112).

It is increasingly recognised that episomal viral replication induces a local DDR activation, perhaps with a greater dependency on ATR phosphorylation of γH2AX as a reflection of replication stress response (107) , but with ATM co-localising at sites of viral replication, especially in differentiated cells where it is thought necessary for viral amplification (108, 113). HRR proteins identified at sites of viral replication include γ 2AX, the MRN complex, RAD51, BRCA1, ATM, ATR, RPA, Chk1 and Chk2 (61, 113-115). The impact of viral replication on the DDR in cancer cells is not yet well understood; especially as carcinogenesis is associated with viral DNA integration into the cellular genome (107, 116).

Intriguingly, despite a recognised activation of many proteins involved in HRR in the presence of HR-HPV, the presence of E7 appears to reduce the ability of cells to repair DNA DSB induced by ionising radiation (54, 82) and this raises the possibility of functional disruption of DNA repair in HPV-associated tumours. A greater understanding of the proficiency of DSB DNA repair within HPV-associated tumours, and the effect this has on treatment efficacy, may lead to novel therapeutic approaches. If tumours demonstrate an impairment of DNA DSB repair, this may be therapeutically exploited.

1.6 Treatment Response in HPV-mediated Gynaecological Cancers

In clinical practice HPV positive cervical and oropharyngeal cancers are recognised as being more radiosensitive than their HPV negative counterparts, and HPV positive vulval tumours have superior outcomes to HPV negative (15-17, 117, 118). The reason for the increased radiation sensitivity in HPV positive OPSCC when compared to HPV negative disease is yet to be established, but OPSCC cell line evidence suggests that impaired DNA DSB repair by HRR could be a contributing factor (83, 119).

Treatment resistance to chemotherapy and radiotherapy remains a problem for this group of tumours and attempts to modulate the DDR to enhance therapeutic response in cervical cancers are ongoing, as reviewed and published by the Author of this thesis (120).

In cervical cancer there is evidence to suggest that genetic polymorphisms of RAD51 and RAD52 are associated with treatment failure (121, 122). One study of irradiated peripheral blood leucocytes from cervical cancer patients with complete response demonstrated increased DNA DSB in response to radiation when compared to those who had an incomplete response (123). Therefore, the response to treatment may not only be dictated by tumour-specific factors but also genetic factors. The identification of patients with inherent resistance to DNA-damaging therapies prior to treatment would allow potential treatment stratification.

ATM is a specific protein of interest with regards to cervical cancer, as impaired levels of transcription have been associated with high miRNA-18a expression and ATM methylation or deletion (124-126). Elevated miRNA -18a correlates with increased cervical cancer radiosensitivity to radical radiation treatment in vitro and in vivo, possibly through ATM repression (124, 125) and a large study correlated high levels of phosphorylated ATM expression in pre-treatment cervical cancer samples with worse loco-regional control and disease-specific survival following radiation with or without chemotherapy (127).

Downstream of ATM, multiple effector proteins have been demonstrated to have generalised pre-treatment upregulation in in locally advanced cervical tumours which are non-complete responders when compared to complete responders, including BRCA1/BRCA2/FANC D2/BRIP 1/RAD51 (17). There appears to be heterogeneity in the levels of DNA repair proteins across cervical tumours, with some cases of cervical cancer demonstrating BRCA1 repression by promoter hypermethylation or other mechanisms (105, 128). Further work is required to evaluate any potential association between tumour HPV status and DDR proficiency in gynaecological cancers. If the presence of HPV influences the ability of tumour cells to repair DNA DSB, comprehensive analysis of DDR protein expression and activation may identify biomarkers of response to therapy (11).

Chapter 2 Thesis Hypotheses and Aims

Evidence to date implicates HR-HPV E6 and E7 proteins in roles abrogating the DNA damage response by overcoming the G1/S cell cycle checkpoint and contributing to replication stress and increased genomic instability, a central hallmark of cancer.

Paradoxically, despite evidence of activation of the DDR by HPV proteins, in vitro evidence from oropharyngeal cancer (OPSCC) secondary cells lines indicates a possible impairment of DNA DSB repair capacity in HPV positive tumours when compared to HPV negative tumours, and this would be in keeping with the clinical radiosensitivity seen in tumours at this anatomical site (129). OPSCC and gynaecological cancers (cervical and vulval cancers) share the aetiological agent of HR-HPV, and the much less studied vulval cancer has a similar proportion of HPV positive and negative cancers to OPSCC (although the aetiological factors for HPV negative cancers differ at these sites; smoking and alcohol in OPSCC and chronic inflammation/lichen sclerosis/smoking in vulval cancer). A hypothesis can therefore be considered that gynaecological HPV-mediated squamous cancers may exhibit impaired DNA DSB repair compared to HPV negative disease, and that this may be associated with impaired activation of the DDR. Hypothesised candidates for impaired activation in cervical tumours are the Fanconi Anaemia pathway and the DNA damage sensor ATM.

There are challenges in assessing the effect of HPV on DNA DSB repair capacity in cells in vitro. It is probable that the effects of ectopically expressed HPV oncoproteins in transformed secondary cell lines will not be entirely reflective of transformed cancer cells in vivo. Such studies give us insights into mechanistic actions of proteins studied but fail to take into account the actions of other genes encoded in the viral genome. There is growing evidence of HPV viral replication centres activating the DDR, and therefore the presence of the viral genome and its status (episomal or integrated) may influence cell fate in response to DNA DSB. It is also likely that cancers will have acquired cell mutations as a consequence of HPV malignant transformation that are not reflected in transfected cell models, and there may well be genetic predispositions or systemic factors at play in cells that have become malignant in the context of HPV persistence. Studies of secondary cancer cell lines abrogate some of these issues, but these models are susceptible to changes due to cell immortalisation and high numbers of passaging in vitro. Studies of DNA DSB repair capability in early passage cancer cells in vitro would provide a better model.

Firstly, this project aims to develop resources to facilitate the investigation into the status of DNA DSB repair in cervical and vulval cancers. An ethically approved study will be set up

to collect biological samples and formalin fixed paraffin embedded (FFPE) tissue will be utilised to develop a tissue microarray (TMA) of cervical tumours, vulval tumours and normal tissues to study protein expression of phosphorylated ATM and FANCD2. This tissue will also be used for RNA extraction to facilitate transcriptional profiling of cervical and vulval cancers via Nanostring technology, with a particular focus on DNA repair genes.

Secondly, fresh tissue will also be collected from study participants to allow the development of primary cells as a novel resource for in vitro study of DNA DSB repair following ionising radiation in cervical and vulval cancer. Such cells will be subject to immunofluorescence studies to assess phosphorylated γ 2AX foci as a marker of DNA DSB frequency pre- and post-irradiation and to determine if RAD51 foci are appropriately formed on induction of DNA damage as a marker of HRR proficiency.

The proposed studies aim to provide novel data on the status of key DDR proteins at the transcriptional, protein expression and functional levels in cervical and vulval cancers (HPV positive and negative). Such studies will help to inform further clinical studies, particularly with regards to potential biomarkers of response to DNA damaging agents and response to novel types of therapy such as PARP inhibitors, which work by exploiting underlying deficiencies in HRR.

Chapter 3 Materials and Methods

3.1 Tissue Microarray Assembly

A tissue microarray (TMA) containing triplicate 0.8mm cores of formalin fixed paraffin embedded tissue (FFPE) was made as previously described by Parsons et al (130) using an Alphametrix MTA-1 manual arrayer. The array was sectioned on a microtome (4µm thickness) and mounted on a glass slide for staining.

3.2 p16, pATM, FANCD2 Immunohistochemistry

P16 immunohistochemistry on TMA sections/cell pellets (fixed in neutral buffered formalin at 4 ^{o}C) was performed and interpreted by M Robinson, consultant histopathologist (Newcastle) as previously published (131). Immunohistochemistry for pATM and FANCD2 was performed on TMA sections following deparaffinisation, rehydration and heat-induced epitope retrieval using the Dako-PT Link machine with low pH antigen retrieval solution (pH 6) at 96^{o}C as per manufacturer's guidance. The EnVision FLEX system (Agilent) was used for benchtop staining as per manufacturers guidance utilising a horseradish peroxidase reaction and DAB+ chromogen visualisation. The primary antibodies FANCD2 (H-300) sc28194 and pATM sc47739 (Santa cruz biotechnology) were diluted to 1:100 with antibody dilutent and 100µl of the antibody solution was incubated on the slide for 30 minutes. EnVision Flex rabbit and mouse linker antibodies were used respectively as provided by manufacturer. Negative staining controls were performed with no primary antibody and positive staining controls with sections expected to express FANCD2 or pATM, with reference to the human protein atlas (normal tonsillar tissue, normal lymph node tissue).

Following immunohistochemistry, the slides were stained with haematoxylin (45 seconds), immersed in acid alcohol (1 second) and Scott's tap water (30 seconds) with a wash in running tap water between each step. The slides were then dehydrated in a series of ethanol (x5) and xylene (x2) and a coverslip mounted with one drop of DPX mountant Sigma-Aldrich). The slides were scanned by an Aperio digital pathology system (Leica biosystems) and visualised using the Aperio ImageScope software for analysis.

3.3 HR-HPV Assessment with RNA in situ Hybridization

RNA in situ hybridisation (ISH) for HR-HPV types 16, 18, 31, 33, 35, 52 and 58 was performed and interpreted by M Robinson, consultant histopathologist (Newcastle) on

TMA sections/formalin-fixed cell pellets using the HPV RNAScope® kit (ABCDbio) as previously published (132).

3.4 mRNA Extraction from Tissue or Cell Culture

From tissue; tissue previously frozen with isopentane and stored at -70 °C (protocol in Appendix A) was thawed or fresh tissue removed from RNAlater® (Thermo Fisher) in a class II safety cabinet. The tissue was dissected into small pieces with a sterile scalpel and trimmed to remove any macroscopic areas not containing epithelium such as blood vessels. Frozen tumour biopsies were taken only from areas of macroscopic identifiable tumour away from the clinical margin. Frozen samples were only included in the study if subsequent histological evaluation from fixed tissue removed concurrently or subsequently confirmed malignancy. Tissue was homogenised in a Precellys®24 homogeniser using a ceramic bead tube (CK 68-R**)** with 700µl of QIAzol® lysis reagent (Qiagen).

From cell culture; the cell pellet was suspended in 700µl QIAzol lysis reagent (Qiagen) and homogenised in a QIAshredder® spin column centrifuged for 2mins at 13000rpm.

RNA was extracted from either tissue or cell culture lysate using the miRNAeasy kit (Qiagen) as per manufacturer's guidance using chloroform to separate RNA into the aqueous phase which was then mixed with 100% ethanol to precipitate the RNA. RNA was then collected and purified using the miRNAeasy spin column with a DNAase digest step. The quality and quantity of RNA eluted in RNAase free water was assessed by the Nanodrop® 1000 spectrophotometer (Thermo Fisher) and the RNA 6000 Nano Kit on the 2100 Bioanalyser (Agilent) as per manufacturer's instructions.

3.5 mRNA Extraction from FFPE Tissue

The paraffin was removed from FFPE tissue cores (1mm diameter and 2-3mm depth) by the addition of 1ml xylene and incubation at 50°C for 3 minutes. The tissue pellet was spun down (centrifuged 13500rpm for 2 minutes) and after incubating on ice for 5 minutes the paraffin/supernatant was removed. The residual xylene was removed by spinning the pellet in 1ml of 100% ethanol (centrifuged at 13500rpm for 2 minutes) and removing the supernatant before allowing any residual ETOH to evaporate. The mRNA was extracted using the RNeasy FFPE kit (Qiagen) as per manufacturer's instructions including tissue lysis by proteinase K in PKD digestion buffer during incubations at 50°C (15minutes) and 80°C (15 minutes). mRNA was then extracted including a DNase incubation, 100% ethanol RNA

precipitation and RNA collection and purification on a RNeasy MinElute spin column. The quality and quantity of RNA eluted in RNAase free water was assessed by the Nanodrop® 1000 spectrophotometer (Thermo Fisher) and the RNA 6000 Nano Kit on the 2100 Bioanalyser (Agilent) as per manufacturer's instructions.

3.6 Multiplex Gene Expression Analysis on the nCounter® SPRINT Profiler

100ng-200ng of input mRNA was used to perform multiplex RNA hybridisation and quantification using a the nCounter® PanCancer Pathways codeset (Nanostring) as per manufacturer's instructions. Data quality control was performed on the n solver 4.0 software.

3.7 Real Time qPCR for HPV 16 E6 and HPV 18 E6

300-500ng input RNA was used for a reverse transcription reaction to produce cDNA for PCR analysis using the QuantiTect® Reverse Transcription Kit (Qiagen) and the Biosystems 2720 thermocycler as per manufacturer's guidance. The quality and quantity of the cDNA was evaluated by the Nanodrop® 1000 spectrophotometer.

Multiplex PCR reactions were performed on the Applied Biosystems® 7500 FAST Dx Real-Time PCR instrument as follows. A total reaction volume (25µl) in each reaction contained 12.5µl Taqman™ Gene Expression Master Mix (Thermo Fisher), 500 nM of each primer, 250 nM of probe, 100-200ng genomic DNA and δH_20 to make up the remaining volume. Primer and FAM-MGB labelled Taqman™ probe for HPV 16 E6 and HPV 18 E6 had previously been designed and custom made (by Applied Biosystems) as described by Schache et al (131). These were multiplexed with VIC-TAMRA labelled beta actin probe (Applied Biosystems) as an endogenous reference gene. Reactions were performed in duplicate for all samples. The HPV16 positive cervical cancer cell line caski (ATCC CRL-1550) and HPV 18 positive Hela (UK Health Protection Agency Culture Collections – 93021013) were used as positive controls and calibrators for the assay. Negative controls wells were plated with no input cDNA.

3.8 Cell Culture

All cell culture was performed with sterile equipment in a class II safety cabinet and flasks incubated at 37 ^oC in 5% CO_2. Secondary cell lines siha (ATCC HTB-35), caski (ATCC CRL-1550), Hela (UK Health Protection Agency Culture Collections – 93021013) and 3T3 fibroblasts (from N Powell, Cardiff) were cultured in 25 cm^2 or 75cm^2 vented flasks (Corning) with Dulbecco's modified Eagle's Medium (DMEM, Gibco) supplemented with

10% fetal bovine serum, 1% L-glutamine, 1% non-essential amino acids and 1% penicillin/streptomycin. Cells were passaged when 70-80% confluent by exposure to trypsin-EDTA, which was quenched by 10ml DMEM and cells pelleted by centrifugation at 1200rpm for 5mins. Cells were then resuspended in fresh medium for transfer to new flask. Prior to use as a feeder layer, 3T3 cells were irradiated with 60Gy in a CellRad x-ray irradiator (Faxitron Bioptics, USA).

3.9 Primary Cell Culture

Biopsy tissue was collected and transported as per the 'Tumour tissue procurement to allow establishment of primary cell cultures for use in Human papillomavirus-mediated cancer research Protocol (Version 1.2)' (Appendix A). The tissue was processed, and primary cells cultured as per 'Primary Cell Culture Protocols for Gynaecological Tissue' (Appendix A).

Spheroid cultures were attempted by seeding cells in ultra-low attachment (ULA) 96 well plates with 100µl of media.

3.10 Cell Immunofluorescence for Pancytokeratin

Cells were seeded onto glass slides and in culture media and left to attach for 48hours. Slides were then washed with ice cold PBS and cells fixed with pre-chilled 100% methanol for 20 minutes at -20^{o}C. Cells were washed and then permeabilised with Triton X-100 (Sigma) 0.5% (in PBS) for 5 minutes and incubated with a blocking solution (5% goat serum and 0.1% Triton X-100, made up in PBS) for 1 hour on a rocker. The slides were then stained sequentially with a pancytokeratin primary antibody solution (ab9377, abcam, 1:200 in blocking solution) and secondly a secondary antibody solution (Alexa-fluor goat anti rabbit 555, Themo Fisher) for 1 hour at room temperature on the rocker (with a wash in between). The secondary antibody was kept in the dark. Negative controls had no primary antibody added to chamber. The slides were then washed with PBS, mounted with Fluoroshield containing DAPI (Sigma) and sealed with clear nail varnish. They were stored in the dark at 4^{o}C until image capture, which was performed as soon as possible on an Olympus BX61 fluorescent microscope with a Photometrics CoolSNAP HQ2 CCD camera. The MicroManager software was used to capture images.

3.11 DNA Extraction

DNA was extracted from cell culture and tissue lysates using the DNeasy Blood and Tissue Kit (Qiagen).

From cell culture; cells at 70-80% confluency were washed and lysed in the tissue culture flask using 200µl PBS, 20µl proteinase K and 200µl AL buffer. 200µl of 100% Ethanol was added to the lysate in the flask and mixed by tapping the flask gently.
From fresh frozen tissue; frozen tissue was thawed in class II safety cabinet and dissected into small pieces with a sterile scalpel. Tissue was homogenised in a Precellys®24 homogeniser in a ceramic bead tube containing 180µL ATL buffer and the homogenate was lysed by the addition of 20µL proteinase k incubated at 56°C until all tissue dissolved. 200µL AL buffer and 200µL 100% ethanol were added and mixed by gentle tapping.
The samples from either source of tissue were then centrifuged in a DNAeasy mini spin column and the DNA was washed and eluted as indicated in the manufacturer's handbook. The quality and quantity of the DNA was assessed on the Nanodrop® 1000 spectrophotometer.

3.12 STR profiling and Mycoplasma Testing

DNA samples were normalised to 2ng/µL and PCR amplification and STR profiling was performed using the Promega GenePrint® 10 system (by A Birss, Institute of Systems, Molecular and Integrative Biology) as per manufacturer's instructions. Results were visualised and interpreted on the Gene Mapper software (Thermo Fisher).

Mycoplasma testing was performed by the Institute of Systems, Molecular and Integrative Biology Mycoplasma testing service at the University of Liverpool utilising the E myco PCR detection kit (Chembio), with 50ng-100ng DNA input for the PCR reaction. The presence of mycoplasma DNA was assessed by agarose gel electrophoresis and imaged on ChemiDoc MP imaging system (Bio-Rad).

3.13 Clonogenic Survival Assays

Clonogenic survival assays were performed by seeding a defined number of cells across multiple in 6 well plates which were subsequently irradiated with 0Gy, 1Gy, 2Gy or 4GY using a CellRad x-ray irradiator (Faxitron Bioptics, USA). After 11-13 days incubation they were stained with 6% glutaraldehyde and 0.5% crystal violet as previously described (129).

Stained colonies were counted on the GelCount colony analyser (Oxford Optronics, Oxford, UK).

3.14 Cell Immunofluorescence

Cell immunofluorescence was performed on primary cells at defined timepoints following exposure to ionising radiation with a method adapted from a Homologous recombination assay protocol written by M Price (Manchester). A defined number of cells (2.5 x 10^3- 10 x $10^{3)}$ were seeded into a chamber slides (Lab-Tek 8 well NUNC coated) with 300µL of the appropriate media. The slides were incubated within a 9cm petri dish in an incubator at 37^{o}C in 5% CO_2 for 24-48hours to allow cell attachment. The chambers were subsequently irradiated with 2Gy-4Gy in a CellRad x-ray irradiator (Faxitron Bioptics, USA). The media was changed post irradiation. At defined timepoints (1hr, 2hr, 4hr, 8hr) the media was removed, the cells were washed with ice cold PBS and cells fixed with pre-chilled 100% methanol for 20 minutes at -20^{o}C. The methanol was washed off with PBS and 300 µL PBS added to the chambers for storage overnight at 4^{o}C.

The cells were permeabilised with Triton X-100 (Sigma) 0.5% (in PBS) for 5 minutes and washed in PBS. Blocking solution was made up from 5% goat serum (Sigma) and 0.1% Triton X-100, made up in PBS. 200µL of block was added to each chamber (kept in a petri dish) and rocked on ice for 1 hour. Primary antibody solutions were made up in block solution. Rabbit anti-RAD51 EPR4030 (Abcam ab133534) 1:1000, mouse anti-phospho-Histone H2A.X (Ser139) clone JBW301 (Millipore, 05-636-I) 1:1000, mouse phosphorylated ATM (santa cruz 47739) 1:500 and rabbit FANCD2 (H:300) (santa cruz 28194) 1:300**.** 200µL primary antibody was added to the chamber wells and the slides were rocked on ice for 1 hour. Negative controls had no primary antibody added to chamber.
Secondary antibodies were made up in blocking solution at a concentration of 1:1000 (Alexa-Fluor donkey anti-mouse 488 and goat anti rabbit 555 Life, Thermo Fisher) and kept away from light by wrapping in foil. After washing the chambers with PBS, secondary antibodies were added to each chamber (200µL) and the chambers were rocked for 1 hour as previously described, covered in foil. After a wash with PBS the chamber walls were removed with forceps and a coverslip as mounted with Fluoroshield containing DAPI (Sigma). The slides were sealed with clear nail varnish and stored in the dark at 4^{o}C until image capture, which was performed as soon as possible on an Olympus BX61 fluorescent microscope with a Photometrics CoolSNAP HQ2 CCD camera. The MicroManager software was used to capture images which were imported into Image J for analysis.

3.15 FFPE Immunofluorescence

Immunofluorescence was performed on 4µm FFPE sections with a method adapted from previous publications assessing RAD51 expression in fixed tissue samples (133). Slides underwent heat-induced epitope retrieval using the Dako-PT Link machine as previously described in section 2.2 except using high pH antigen retrieval solution (pH 9). Slides were permeabilised in 0.2% Triton™ X-100 (made up in PBS) for 20 minutes, washed with PBS and incubated with 100-200µL (enough to cover area) of 1:10000 solution of DNase I (Omega bio-tek) made up in PBS at 37 ^{o}C in a humidified chamber for 1 hour. The slides were then washed with PBS and incubated with 200µl of blocking solution (1% Bovine serum albumin and 2% fetal bovine serum in PBS) for 30mins at room temperature.

The slides were then stained with 200µl of geminin antibody (Monoclonal anti-Geminin clone 1A8 WH0051053M1, Sigma, 1:xxx in blocking solution) for one hour on the benchtop, followed by a PBS wash and staining with 200µl of secondary antibodies (Alexa-fluor donkey anti-mouse 488 1:1000, Thermo Fisher) for 1 hour in the dark. The slides were then washed and fixed in 4% paraformaldehyde for 15mins in a fume hood. The slides were then washed and stained with the RAD51 antibody (Abcam ab133534, 1:500 in blocking solution) and washed and stained with the secondary antibody (Alexa-fluor goat anti rabbit 555 1:1000, Thermo Fisher) in the same way as the geminin staining. The cells were then again fixed with 4% paraformaldehyde and a coverslip mounted with Fluoroshield containing DAPI (Sigma) and sealed with clear nail varnish.

Chapter 4 – Tissue Microarray Immunohistochemistry

4.1.TMA Assembly and Core Evaluation

Archived FFPE cervical and vulval tumour tissue blocks were obtained under the study 'Tumour tissue procurement to allow establishment of primary cell cultures for use in Human papillomavirus-mediated cancer research' (protocol version 1.2, Appendix A). Archived normal cervical tissue blocks were obtained from the Liverpool Tissue Bank. A summary of tissue samples used with clinicopathological data is found in Appendix B. These tissue blocks were from pre-treatment biopsies and surgical specimens, depending on the availability of each. A representative hematoxylin and eosin (H&E) stained slide by for each block was reviewed and marked (tumour tissue, normal tissue) by a histopathologist with expertise in gynaecological cancers (S Ruthven). Tumour, adjacent normal and normal cervical tissues were then sampled in triplicate across the TMA as 0.8mm punches using the Alphametrix MTA-1 manual arrayer. The array was sectioned on a microtome (4um thickness) and fixed onto a glass slide for staining.

A section of each TMA utilised underwent H&E staining and p16 staining which was reviewed by histopathologists with the appropriate expertise (S Ruthven, M Robinson) to confirm contents of each tissue core and determine p16 status as a surrogate marker of HPV status. A representative section was also utilised to perform HR-HPV RNA hybridisation (M Robinson) to assess for HR-HPV presence (HPV ACDBio RNAScope probe HPV 16, 18, 31, 33, 35, 52 and 58). There was a higher incidence of HPV negative cervical cancer (30%) than would be expected, with the literature estimating the rate of cervical cancers testing negative for HPV between 5.5-11%, and many of these representing false negatives (134). This highlights the importance of multi-modality HPV testing, such as the use of HPV-DNA ISH in addition to HPV RNA ISH, which may reduce false negative rates.

For the purposes of this analysis, analyses for HPV status were performed using both p16 and HPV RNA status separately, as three of the six cervical cancers with negative HPV RNA ISH were p16 positive, and p16 has previously been utilised as a surrogate marker of HR-HPV status in gynaecological cancers (106). It is possible that RNA degradation during fixation led to false negative HPV RNA ISH, or that genotypes not included in the panel were present.

4.2 ATM

4.2.1 ATM in HPV mediated Gynaecological Cancers

The first protein investigated by immunohistochemistry in this study was phosphorylated ATM (serine 1981) (pATM). In addition to the assembled TMA containing cervical and vulval tumour tissue, TMA sections were available within the group from previous work (A Schache, F Greaney) containing oropharyngeal squamous cancer (OPSCC) samples, constructed in the same manner as the gynaecological TMA but with 1mm tumour cores (132, 135). TMA sections containing OPSCC cores were therefore also stained to strengthen the comparison of HPV positive versus HPV negative tumours and also facilitate cross-comparison of results at a third anatomical site.

ATM is of particular interest in HPV-associated gynaecological tumours as there is evidence to suggest that expression may be supressed in a proportion of cervical tumours. Mechanisms for this include *ATM* promoter methylation and LOH on chromosome 11q22-23 (126). A study of a large sample set of cervical cancers by Indra et al demonstrated promoter methylation in up to 36% of cases and deletion of *ATM* in 31% cases (126). This resulted in just under half (49%) of cases having at least one form of *ATM* genetic alteration, and 16.4% having biallelic methylation/deletion. The study demonstrated a correlation between *ATM* methylation/deletion and reduced *ATM* transcripts and ATM protein expression in a small subset of tumours. The effect on levels of activated ATM (phosphorylated at serine 1981) were not reported, although comment was made that the staining of pATM was predominantly cytoplasmic, which may be considered surprising given its nuclear role in coordinating the DDR. Survival curves in the large cohort of tumours indicated that ATM methylation or deletion were associated with worse overall survival (126). The samples included a wide range of disease stage (I-IV) and treatment schedules for the patients are not known.

A second study in a large cohort of locally advanced cervical cancers (treated with radiation +/- chemotherapy) by Roossink et al assessed immunohistochemical expression specifically of ATM phosphorylated at serine 1981 (pATM) (127). This study classified pATM expression as high or low with an approximately 50% split between the two cohorts. High levels of pATM were associated with a worse outcome in terms of disease-specific survival. ATM methylation or deletion status were not assessed in this study.

These two studies present a discrepancy between the predominant sub-cellular localisation of pATM in cervical cancer; the epitomics EP1890Y antibody demonstrated nuclear pATM predominance, with the santa-cruz sc47739 antibody reported as showing a predominantly cytoplasmic stain (126, 127). Phosphorylated ATM is thought to exist within both cellular compartments as a dimeric form in the cytoplasm which is monomerised and transported into the nucleus to promote DNA repair following DNA damage (136). The roles of cytoplasmic pATM are still under investigation and include insulin signalling, mitochondrial function, and triggering of apoptosis via interaction with Akt (137, 138). Therefore, cytoplasmic ATM could have a role in determining cell fate via apoptosis on exposure to DNA damaging therapies independently of DNA repair. The two studies also demonstrate different associations with ATM and pATM and disease-specific survival. The Indra et al study indicated that *ATM* methylation or deletion (which was associated with low *ATM* transcription/expression) was associated with worse survival, and the Roossink et al study indicating that low pATM was associated with better survival. A poor/inverse correlation between *ATM* gene expression and ATM activation/phosphorylation may explain these findings.

On review of the literature of squamous tumours at other HPV-related sites, there appears to be a common theme of ATM gene alteration by methylation and LOH at Chromosome 11q22-23. Studies of OPSCC have demonstrated ATM promoter methylation in 25%-42% of cases studied (139, 140), and a further study of HNSCC (the majority from the oropharynx) demonstrated ATM loss in 60% of cases (141). Deletions of ATM have also been seen in anal cancer, and loss of 11q identified as a feature of vulval cancers (142-144). To my knowledge, no relationship between ATM/pATM status and tumour HPV status has been studied at any anatomical site.

This study aims to clarify the status of phosphorylated ATM in untreated cancer samples from the cervix, vulva and oropharynx, and normal control tissues form the same anatomical locations. I have chosen to use a p-ATM antibody which demonstrated both nuclear and cytoplasmic staining during staining protocol optimisation. This will provide novel data for cervical, vulval and oropharyngeal tumours utilising this antibody, and will be comparable to the findings of Roossink et al who performed the largest study of pATM to date in cervical cancer using an alternative antibody. This data should clarify the predominant staining pattern for pATM in these tumours (nuclear or cytoplasmic), how expression levels of pATM correlate with (matched) normal tissues, HPV status, clinicopathological data and outcome data. This analysis will also allow patterns of pATM

expression to be correlated with ATM transcription when paired samples are analysed by the Nanostring technology in a later study.

4.2.2 Phosphorylated ATM Immunohistochemistry Optimisation and Staining

Optimisation of antibody staining was performed and a concentration of 1:100 selected to give representative staining with minimum background. Multiple tissue microarray sections were stained by immunohistochemistry using the Dako Flex System as per manufacturers guidance. The stained slides were scanned electronically using the Aperio system for scoring by trained reviewers (S Ruthven, L Cossar, G Gill).

4.2.3 Development of pATM Immunohistochemistry Scoring System

No standardised scoring system exists for evaluation of pATM, and therefore a scoring system was devised for this study (Table 4.1). Previous studies in cervical cancer have utilised scoring systems combining two scores (staining intensity score and percentage of epithelial/tumour cells stained) to give an overall staining level. A novel scoring system was designed to separately evaluate nuclear and cytoplasmic pATM scoring for analysis, using staining intensity and percentage of tumour cell stained. The scores for both cellular compartments were then used to give an overall level of pATM expression. After recognising that the tissue samples demonstrated overall high percentages of pATM staining, the categories for percentage staining scores were selected to separate the data into approximately equal tertiles. Representative tissue cores for nuclear and cytoplasmic staining intensity are found in Figure 4.1. Each core was scored by independently by two assessors and where there was discrepancy the cores were re-reviewed, and a consensus reached between two reviewers. The mode of intensity and percentage scores for nuclear and cytoplasmic staining across the available duplicate/triplicate cores were used for the final analysis. Where no mode was available a mean was calculated and rounded up to the nearest integer.

Table 4.1 pATM immunohistochemistry scoring system

Nuclear Intensity		**Nuclear percentage**		**Total Nuclear Score**	**Overall Score**
1	None/low	1	0-50%	2-6	4-12
2	Moderate	2	60-80%		
3	High	3	90-100%		
Cytoplasmic Intensity		**Cytoplasmic percentage**		**Total Cytoplasmic Score**	
1	None/Low	1	0-40%	2-6	
2	Moderate	2	50-80%		
3	High	3	90-100%		

4.2.4 – Summary of Cores Analysed

The total number of tissue samples that were available for analysis is summarised in Table 4.2. A degree of core loss occurred during the sectioning and staining of the TMA. Data was available from at least two cores in 70% of tumour cases.

Table 4.2 Summary of Clinical Samples Analysed for pATM Expression

Anatomical Location	**Tumour**			**Normal**
	HPV DNA/RNA ISH positive	HPV DNA/RNA ISH negative	HPV DNA/RNA status unknown	
Cervix	14	6	0	33
Vulva	3	21	0	19
Oropharynx	43	42	17	31

4.2.5 Comparative Expression of pATM in Cervical, Vulval and Oropharyngeal Cancers

The predominant staining pattern seen in cervical cancers was nuclear, with 85% of tumours having a higher nuclear score than cytoplasmic score. This was also the case in

65% of OPSCC. Vulval tumours had more cytoplasmic staining relative to the other tumour types, with only 48% of tumours having a greater nuclear pATM staining score than cytoplasmic.

The mean values for overall and nuclear staining for pATM in tumour and normal tissues are shown in Figure 4.2 and Figure 4.3. There was a significant increase in the overall and nuclear staining values in OPSCC and cervical cancers compared to normal tissue, but no changes seen in the vulval cancer cohort. No tumour demonstrated significantly altered cytoplasmic staining values when compared to normal tissue.

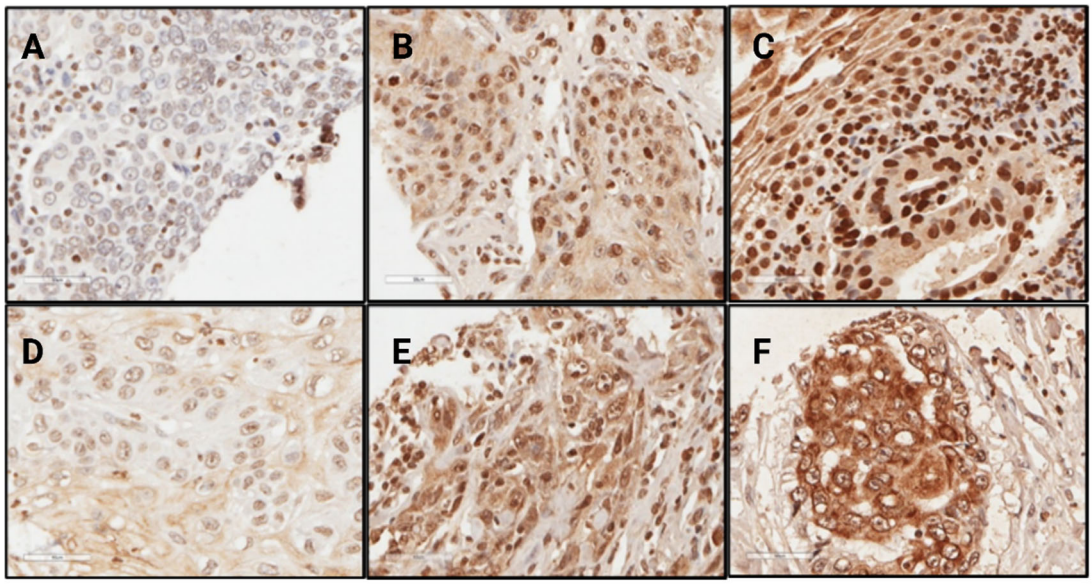


Figure 4.1 Microscopy images demonstrating typical appearances of nuclear pATM staining intensity 1, 2 and 3 (A, B and C) and cytoplasmic pATM staining intensity 1, 2 and 3 (D, E and F).

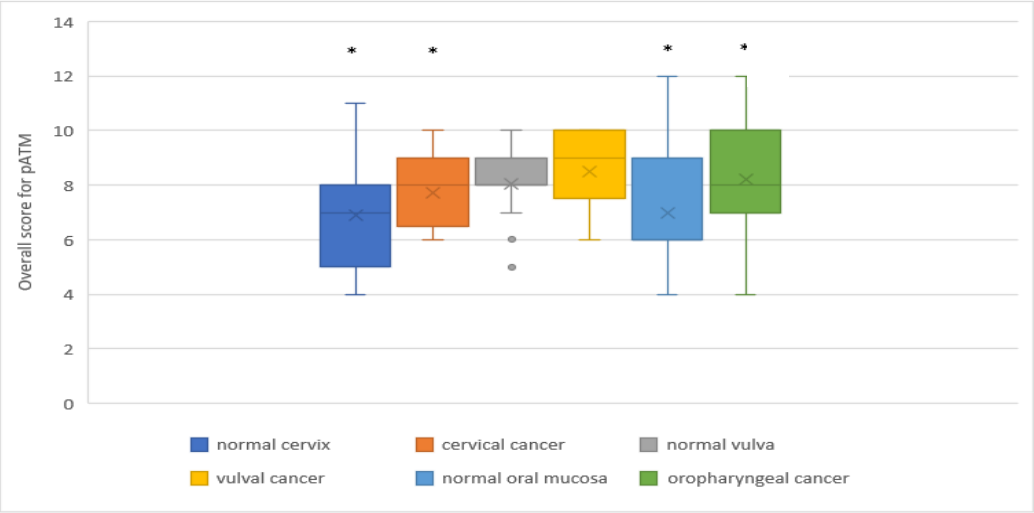


Figure 4.2 Overall pATM staining scores displayed by tissue type *denotes statistical significantly change (independent t test) between tumour tissue and normal tissue from site of origin (p= 0.05 for cervical cancer, p<0.001 for OPSCC)

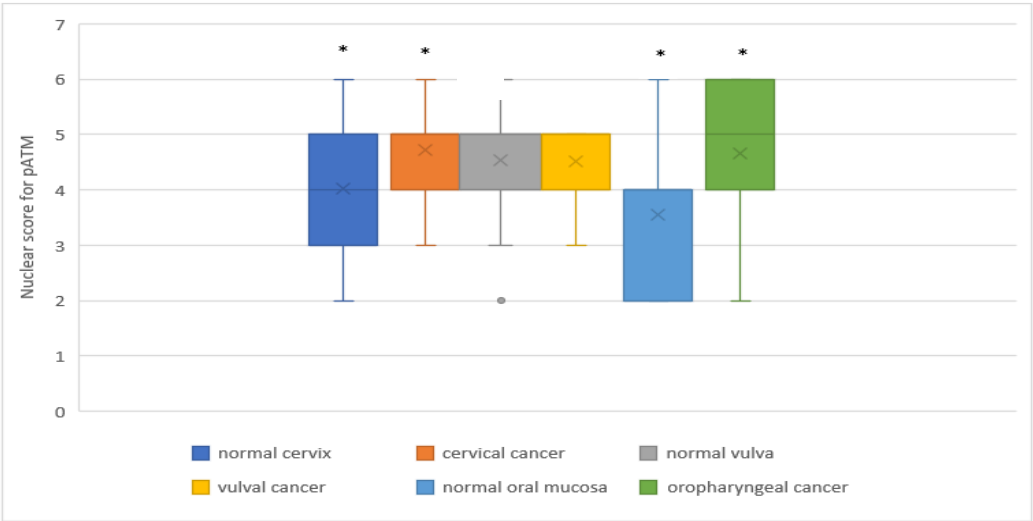


Figure 4.3 Nuclear pATM staining scores displayed by tissue type *denotes statistical significantly change (independent t test) between tumour tissue and normal tissue from site of origin (p= 0.016 for cervical cancer, p<0.01 for OPSCC)

Further analysis was performed on the matched samples (tumour and adjacent normal) available in the gynaecological cancer cohort to determine relative expression (12 cervical cancers, 16 vulval cancers). Overall pATM scores were lower in 33% of cervical tumours and 19% of vulval tumours compared to tissue of origin.

A shared feature of the cervical and OPSCC is a high proportion of HPV-mediated cancers when compared to the vulval cohort (Table 4.2). The possibility that overall increases in pATM staining in these cancers could be related to HPV status was examined. When all HPV positive tumours were analysed in comparison to all HPV negative tumours, there was no significant different in overall pATM staining scores. However, when nuclear and cytoplasmic scores are analysed independently, there is a significant increase in nuclear pATM scores and a significant decrease in cytoplasmic pATM scores when HPV positive tumours are compared to HPV negative ones ($p = 0.003$ and $p = 0.002$ respectively). This pattern is also seen when the OPSCC cohort is analysed separately by HPV status although the decrease in cytoplasmic staining does not reach statistical significance ($p = 0.04$ and $p = 0.139$).

4.2.6 Association of pATM Expression with Clinical Outcomes

To facilitate statistical analysis of the pATM staining data with clinical outcomes, a simplified version of staining score was devised. Such an approach has been previously utilised in cervical studies of pATM, having been adapted from OPSCC studies evaluating other proteins of interest (126, 145). In addition to applying these categories to the overall staining scores, it was also applied to the total nuclear and total cytoplasmic staining as seen in Table 4.3. Categories were assigned after a review of the spread of the original scoring data (Appendix B). Two categories (High, Low) were assigned unless the data exhibited a normal distribution in which case three categories (Low, Moderate, High) were used to preserve the integrity of the data. This scoring system was applied to the mode/median score for each tissue sample as before.

Table 4.3 – Simplified pATM Immunohistochemistry Scoring System applied for data analysis

Total Nuclear Score		**Overall Score**	
2-4	Low	4-6	Low
5-6	High	7-9	Moderate
Total Cytoplasmic Score			
2-3	Low	10-12	High
4-6	High		

The simplified immunohistochemical staining scores were analysed in the SPSS statistical software program, utilising Chi squared cross-tabulations and survival outcome data analysed by Kaplan Meir and Cox regression analysis. There were no significant associations between total nuclear, total cytoplasmic or overall pATM staining and sex of the patient, stage of disease, alcohol consumption or smoking in the OPSCC cohort. The majority of the patients were smokers, drank more alcohol than UK guidelines and were of stage 3 or 4 disease as classified by TNM 7 (146). The only significant association between pATM staining and stage of the disease in the vulval or cervical cancer cohorts was that the more advanced cervical cancers (stage 3 or 4, n=3) had a significantly higher total cytoplasmic score p=0.009. All of these cases were HPV negative, and therefore this could be a reflection of HPV status given the proposed relationship between HPV negative tumours and increased cytoplasmic pATM.

When all tumour types were analysed together, there was a significant association between lower total pATM nuclear scores and disease recurrence ($p = 0.034$). When OPSCC and gynaecological tumour cohorts were analysed separately the same association was identified ($p = 0.007$, $p = 0.035$ respectively). There was also a significant association between HPV negative disease and disease recurrence in both the OPSCC and gynaecological cohort ($p=0.004$, $p=0.011$). There was no association with recurrence with stage, alcohol consumption, smoking history, sex, or age of patients. The lack of association between stage and recurrence is likely a reflection of the small numbers of high stage disease in the gynaecological tumours, and the low numbers of early stage disease in the OPSCC cohort.

Overall survival was analysed for the OPSCC group only due to the smaller numbers and heterogenous cases/treatment schedules in the gynaecological cohort. There was no association found between overall or nuclear pATM scores and disease-specific survival, Figure 4.4. As previously reported, HPV status was found to be significantly associated with disease-specific survival, with HPV positive disease (determined by p16 or HPV ISH) having significantly greater survival ($p=0.002$, $p<0.001$) (Figure 4.5).

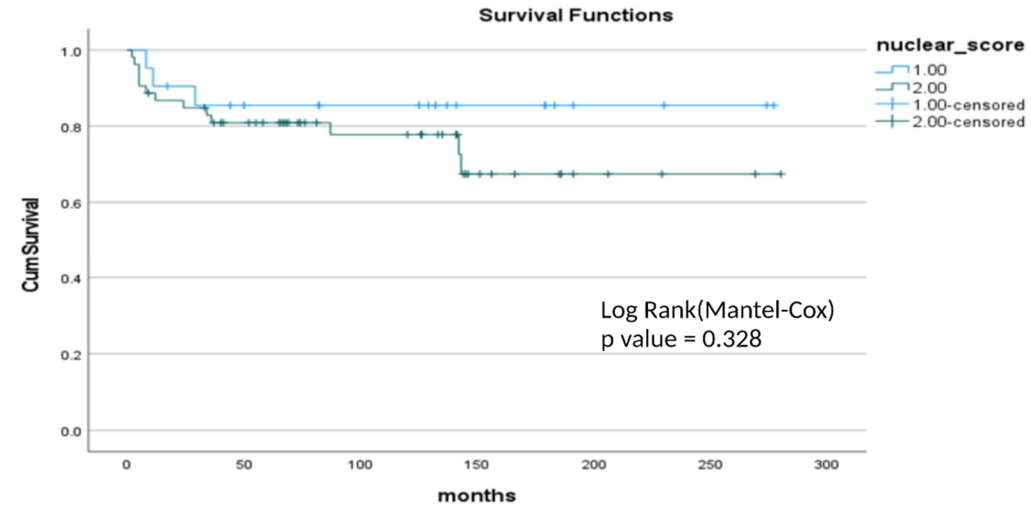


Figure 4.4 Kaplan-Meier Plot of disease-specific survival in OPSCC by pATM nuclear scoring (1=low, 2=high)

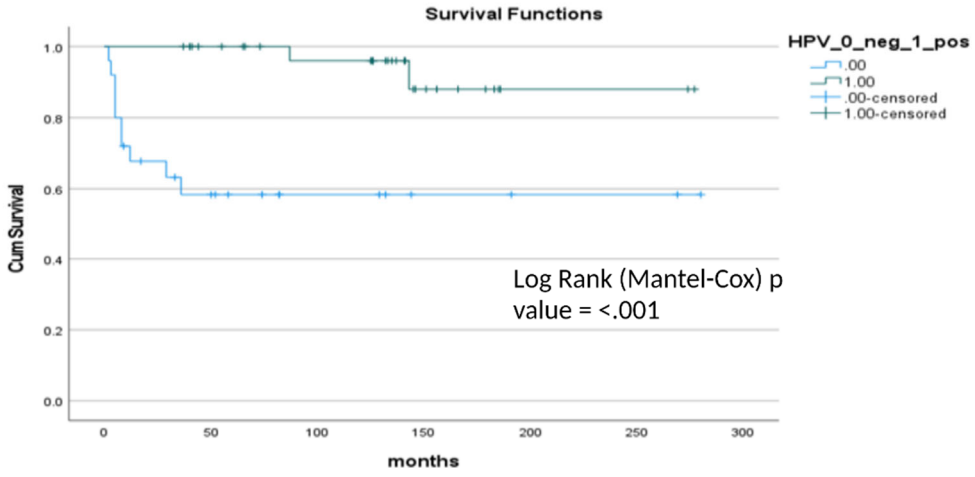


Figure 4.5 Kaplan-Meier Plot of disease-specific survival in OPSCC by HPV status (0=negative, 1=positive)

Cox regression analysis was performed with the variables of disease stage, HPV status and pATM status to identify the impact of nuclear pATM levels on survival (Tables 4.4 and 4.5). identified nuclear pATM staining as a significant independent variable in survival outcomes in the group treated with (chemo)radiotherapy as part of their primary therapy with low nuclear pATM scores being associated with increased survival (p = 0.025, HR 19), and a trend towards this finding in all OPSCC, irrespective of treatment received (p=0.073, HR 4.1).

Table 4.4 - Cox regression analysis of OPSCC survival in patients receiving radiation therapy with or without chemotherapy

	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)
HPV status	-5.507	1.928	8.159	1	0.004	0.004	0
Stage	-2.888	1.301	4.932	1	0.026	0.056	0.004
Nuclear score	2.976	1.325	5.04	1	0.025	19.606	1.459

Table 4.5 - Cox regression analysis of OPSCC survival in all OPSCC patients

	B	SE	Wald	df	Sig.	Exp(B)	95.0% CI for Exp(B)
HPV status	-2.555	0.808	10	1	0.002	0.078	0.016
Stage	-0.141	0.805	0.031	1	0.861	0.869	0.179
Nuclear score	1.421	0.793	3.212	1	0.073	4.141	0.876

4.2.7 Discussion

This study has added to the current knowledge of the status of ATM activation in cervical, vulval and OPSCC cancers, not only by assessing levels of phosphorylated ATM, but also in evaluating the distribution of pATM between subcellular compartments. Overall and nuclear levels of pATM are elevated in cervical and OPSCC, but not in the vulval cancer cohort. The increased number of HPV-mediated cancers in the cervical and OPSCC cohorts

do not account for the increased overall level of pATM staining in these tumours, but analysis by HPV status does demonstrate an increased shift to nuclear pATM and loss of cytoplasmic pATM in HPV positive tumours. This could possibly be due to increased nuclear shuttling of pATM in this context. Previous studies have identified ATM nuclear activation by HR-HPV viruses (108, 109). Possible mechanisms for this have been postulated in recent publications such as ATM activation at viral replication centres or the accumulation of unrepaired DNA damage due to impaired cellular DNA repair kinetics in the presence of HPV (61, 113, 147, 148) Recently, a similar study on cervical cancer also evaluated pATM nuclear status, demonstrating higher nuclear pATM in cervical cancer than normal cervix and a trend towards higher nuclear pATM in HPV positive tumours, corroborating the findings of this analysis (149).

This study also demonstrates that increase in overall pATM is not universal during the malignant transformation from normal cervix and cervical cancer, with a third of cervical tumours demonstrating a lower overall pATM staining score. This is consistent with the evidence in the literature of ATM loss by promoter methylation or deletion in a subset of tumours (126), although it is possible (but not likely) that these tumours are exhibiting lower levels of DDR activation than surrounding tissues due to lower endogenous DNA damage. These findings lend credence to the hypothesis that pATM status of tumours, in particular the expression of nuclear pATM, can be influenced by transcriptional activity and HPV presence.

HPV status is the strongest predictive factor of disease-specific survival for OPSCC, and this is widely acknowledged in the literature (150). A possible mechanism for this is increased radiosensitivity linked to delayed DNA repair kinetics (129). It seems unlikely that the increased radiosensitivity of HPV positive OPSCC tumours is mediated by loss of pATM function, given the increased levels of nuclear pATM identified in this population, although this study was not able to assess the dynamic response of ATM to exogenous DNA damage or completion of DNA DSB repair. This study highlighted that low pATM levels have been associated with an increased rate of recurrence in both gynaecological and OPSCC cohorts, and it is possible that this is a reflection of the HPV status of the tumours, as HPV negative tumours are more likely to have low nuclear pATM levels. However, this did not translate into an inferior survival in OPSCC patients with low nuclear pATM levels at baseline, in fact there is a trend towards improved survival in OPSCC with low nuclear pATM. This can be explained by the results of the cox regression analysis identifying nuclear pATM staining as

a significant independent variable in survival outcomes, with low nuclear pATM favouring better survival, despite its association with HPV status. Low pATM may be associated with HPV negative cancers with a worse prognosis, but if a tumour has low pATM it may be more radiosensitive due to loss of DDR activation and DNA DSB repair. This is also in keeping with the data in cervical cancer by Roossink et al which identified better outcomes in patients receiving chemoradiotherapy for locally advanced cervical cancer who had low nuclear pATM expression within a cohort of HPV positive tumours (127).

To further understand the dynamics and functionality of pATM in gynaecological tumours it would be useful to determine the relationship between transcription of ATM and pATM expression in untreated tissues, and this will be taken forward in the planned Nanostring study evaluating ATM transcripts in RNA extracted from the same FFPE tissue blocks as utilised for the TMA. An assessment of dynamic DNA repair following radiation would also be useful in cervical and gynaecological tumour cell lines to determine if low baseline pATM results in delayed repair kinetics.

4.3 FANCD2

4.3.1 FANCD2 in HPV mediated Gynaecological Cancers

The second protein selected for evaluation by immunohistochemistry was FANCD2, a critical effector of the Fanconi Anaemia (FA) pathway of DNA repair proteins. Loss of functionality of this pathway due to the biallelic loss of a FA gene (Fanconi anaemia syndrome) has been associated with a high risk of anogenital squamous cell cancers including vulval and cervical cancer (151, 152). Previous mouse model studies have demonstrated that lack of FA pathway activity is associated with increase development of HPV-mediated cancers (94, 153). Oropharyngeal cancer shares HPV-mediated aetiology with cervical and vulval cancer and a prior study demonstrated a loss of FANCD2 immunohistochemistry staining during malignant transformation in HPV positive tonsillar cancers (154). This is possibly associated with Chromosome 3p25 loss, which is frequently seen in oral cancers (155) Another study demonstrated no evidence of reduced FANCD2 transcription in oral cancers and therefore other mechanisms may also be responsible for lower FANCD2 protein expression (155, 156). Whether FANCD2 loss exists in gynaecological HPV-mediated tumours has not been established, although Chromosome 3p25 loss is also recognised in CIN, VIN, and cervical cancer (157-159). Santegoets et al described increased FANCD2 transcription in HR-HPV associated VIN when compared to normal vulva, but vulval cancers were not included in this study (106). A study into the status of FANCD2 in cervical

and vulval cancers could lead to new insights into the development and treatment of these tumours. Given the key role of the FA pathway in repairing damage induced by chemoradiotherapy, FANCD2 could potentially be a biomarker of response to treatment, as suggested by work from Balacescu et al in locally advanced cervical tumours (17).

The actions of FANCD2 are widely acknowledged to be nuclear with regards to the maintenance of genomic integrity, but FANCD2 also exists within the cytoplasmic compartment. The presence of cytoplasmic staining has previously been associated with a more favourable prognosis in breast and ovarian cancer (160, 161). The study in breast cancer patients estimated the percentage of cells expressing cytoplasmic FANCD2, whilst the study in ovarian cancer chose to look at cytoplasmic staining intensity. This study of FANCD2 expression will therefore seek to characterise the protein levels of FANCD2 in both nuclear and cytoplasmic compartments in cervical and vulval cancers, as assessed by percentage of stained cells and staining intensity. The second aim will be to determine if levels of FANCD2 expression during malignant transformation in OPSCC are associated with clinical outcome data.

4.3.2 FANCD2 Immunohistochemistry optimisation and staining

Multiple tissue microarrays were stained with a rabbit polyclonal FANCD2 antibody (Sc - 21894), which had previously been utilised in a publication evaluating FANCD2 expression in breast cancer (160). This antibody demonstrates both nuclear and cytoplasmic FANCD2 staining.

Antibody optimisation was undertaken using the DAKO Envision Flex system as per manufacturer's instructions and a dilution of 1:100 was selected as the optimal antibody concentration to provide sufficient staining intensity and to minimize background staining (Figure 4.6).

4.3.3 Development of a FANCD2 Immunohistochemistry Scoring System

There is no standardised staining system for FANCD2 immunohistochemical evaluation, and therefore a scoring system was devised to separately evaluate nuclear and cytoplasmic FANCD2 to determine if subcellular localisation of this protein may be of relevance in addition to total protein expressed. The scoring was based on staining intensity and percentage of epithelial/tumour cells stained as demonstrated in Table 4.6. The nuclear

and cytoplasmic score were then combined to demonstrate an overall FANCD2 expression score.

Table 4.6 – FANCD2 immunohistochemistry scoring system

Nuclear Intensity		**Nuclear percentage**		**Total Nuclear Score**	**Overall Score**
1	None/low	1	0-30%	2-6	4-12
2	Moderate	2	40-60%		
3	High	3	70-100%		
Cytoplasmic Intensity		**Cytoplasmic percentage**		**Total Cytoplasmic Score**	
1	None	1	0%	2-6	
2	Low/moderate	2	10-70%		
3	High	3	80-100%		

Each core was scored independently by two assessors (L Cossar, G Gill or S Ruthven) and where there was discrepancy between scores the cores were re-reviewed, and a consensus reached between two reviewers.

4.3.4 – Summary of Cores Analysed

A total number of 183 tissue samples were available for analysis as summarised in Table 4.7. The normal samples were adjacent normal (cervix and vulva) and a set of normal cervix controls. Each clinical case was sampled in triplicate across the TMA, but some core loss occurred during the sectioning and staining of the TMA. Data was available from at least two cores in 82% of tumour cases and 42% of normal tissue samples. The mode of the scores across the duplicate/triplicate cores was used for the final analysis. Where no mode was available a mean was calculated and rounded up to the nearest integer.

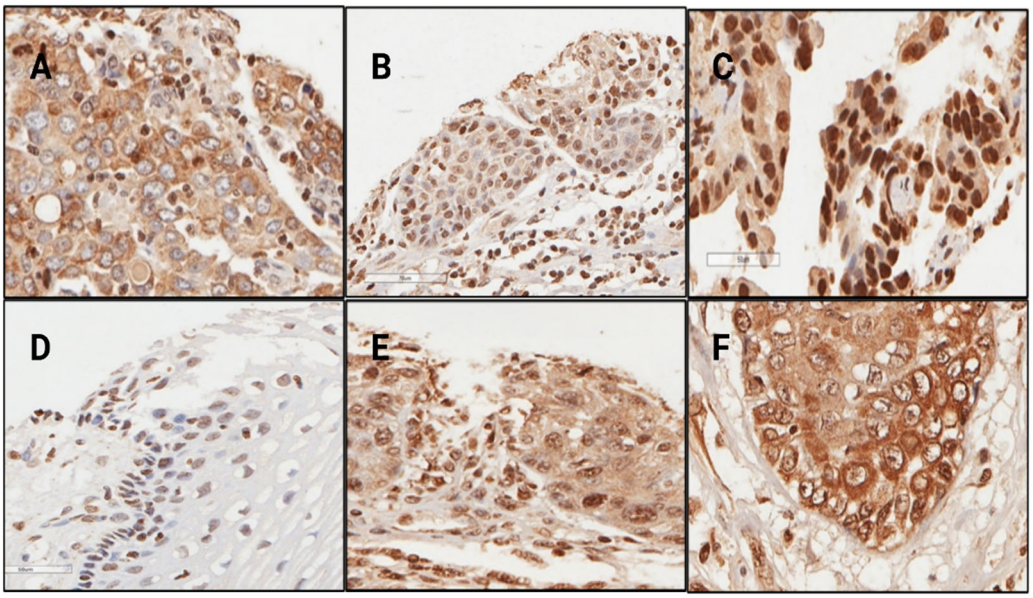


Figure 4.6 Microscopy images demonstrating typical appearances of nuclear FANCD2 staining intensity 1, 2 and 3 (A, B and C) and cytoplasmic FANCD2 staining intensity 1, 2 and 3 (D, E and F).

Table 4.7 – Summary of Clinical Samples available for FANCD2 Immunohistochemical Analysis

Anatomical Location	Tumour			Normal
	HPV DNA/RNA ISH positive	HPV DNA/RNA ISH negative	HPV DNA/RNA status unknown	
Cervix	11	6	0	32
Vulva	3	22	0	19
Oropharynx	47	37	5	0

4.3.5 Comparative Expression of FANCD2 in Cervical, Vulval and Oropharyngeal Cancers

Normal tissues (cervix and vulval) both demonstrated FANCD2 staining with a nuclear predominance in the majority of cases (74%, 84% respectively). The relative degree of cytoplasmic staining increased with malignant transformation in both gynaecological tumour groups with cervical cancers demonstrating 47% nuclear predominance, 29% cytoplasmic predominance. Vulval tumours similarly had 40% of tumours with higher nuclear FANCD2 staining scores and 32% with higher cytoplasmic staining scores. Due to constraints on TMA sections available, there was no normal OPSCC tissue stained, but the OPSCC samples demonstrated low levels of relative nuclear FANCD2 staining with 24% having higher nuclear staining scores and 55% having higher cytoplasmic staining scores. A similar trend was seen in both the HPV negative and HPV positive OPSCC cohorts.

When compared to normal tissues, cervical cancer demonstrated a significantly higher FANCD2 scoring than normal cervical tissue (Figure 4.7, 4.8, 4.9), and this was found to be the case in both nuclear and cytoplasmic compartments. Vulval tumours demonstrated no change in overall FANCD2 scoring, but when analysed by compartment staining, vulval tumours had significantly lower nuclear staining and significantly higher cytoplasmic staining than normal vulval controls. Overall, nuclear, and cytoplasmic FANCD2 levels in OPSCC were similar to those observed in vulval tumours.

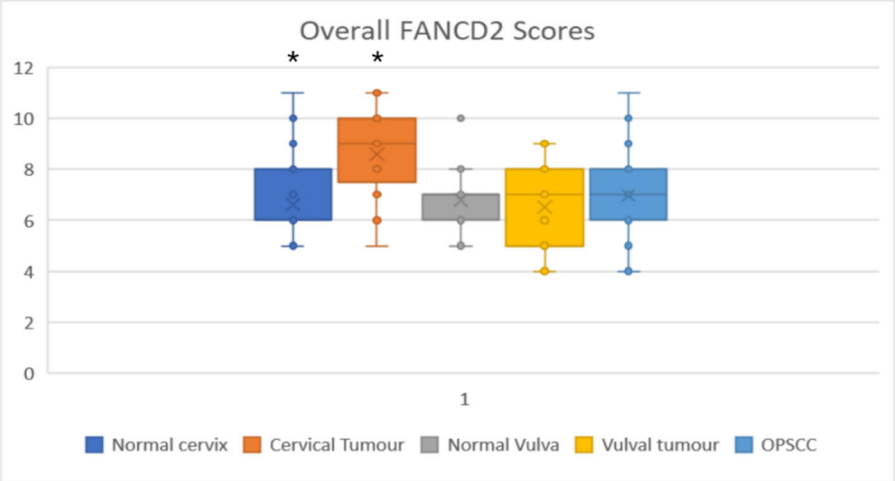


Figure 4.7 Overall FANCD2 staining scores displayed by tissue type. * denotes statistical significantly change (independent t test) between tumour tissue and normal tissue from site of origin ($p < 0.001$)

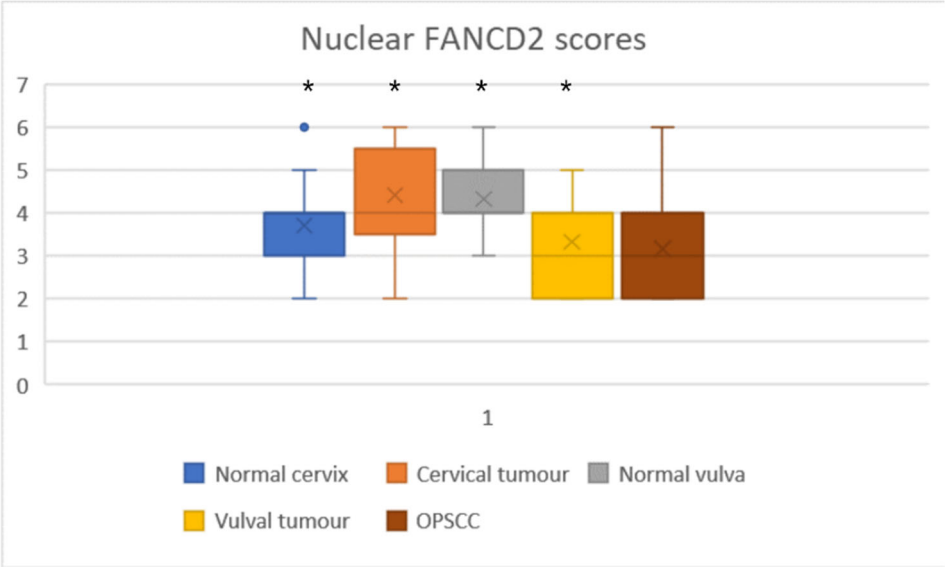


Figure 4.8 Nuclear FANCD2 staining scores displayed by tissue type. * denotes statistical significantly change (independent t test) between tumour tissue and normal tissue from site of origin (cervix p=0.045 vulval p=0.002)

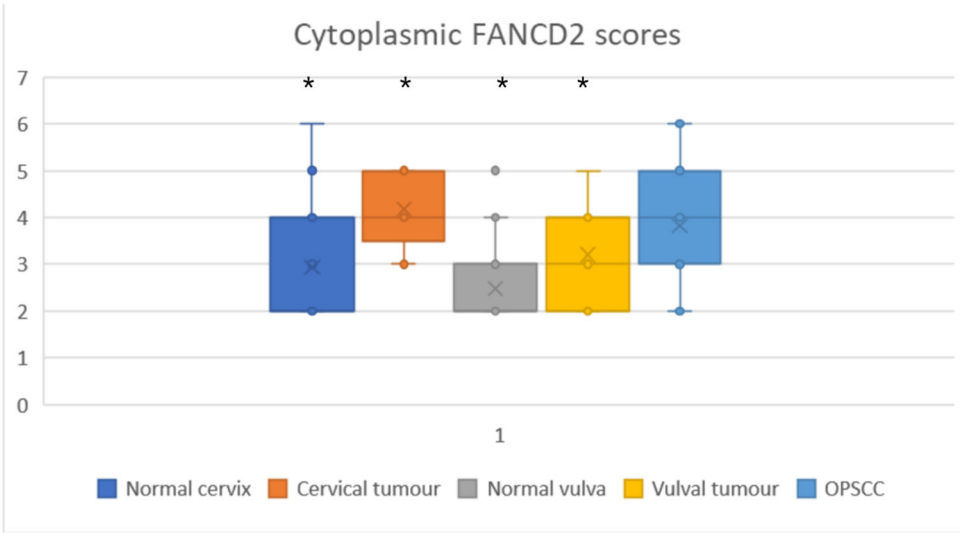


Figure 4.9 Cytoplasmic FANCD2 staining scores displayed by tissue type. *denotes statistical significantly change (independent t test) between tumour tissue and normal tissue from site of origin (cervix p <0.001, vulval p=0.014)

There were no significant differences in FANCD2 staining scores between HPV negative and HPV positive tumours in the overall cohort or when analysed by tumour group. There were no differences in FANCD2 staining between normal cervix from non-malignant hysterectomies and normal cervix taken adjacent to cervical tumour formation and therefore no indication of a relative loss of FANCD2 predisposing to the development of cervical tumours.

4.3.6 Association of FANCD2 expression with clinical outcomes

To facilitate further analysis of FANCD2 staining, a simplified version of the score was devised to enable the division of the scores into two categories 'Low' and 'High' FANCD2 intensity, percentage, or overall expression (spread of scoring data available in Appendix B). Categorising staining scores to enable cross-tabulation analysis was previously used in the study of FANCD2 in breast cancer by Rudland et al (160). The scoring system was applied to the mode/median score for each tissue sample as demonstrated in Table 4.8.

Table 4.8 Simplified FANCD2 immunohistochemical scoring system used for final analysis

Total Nuclear/Cytoplasmic Score		**Overall Score**	
2-3	Low	4-6	Low
4-6	High	7-8	Moderate
		9-12	High

Analysis of changes in FANCD2 expression between the samples analysed were performed in SPSS statistical software by Pearson chi-squared crosstabulation and Fishers exact test.

There was no statistically significant difference in FANCD2 scoring according to age, stage, or HPV status in cervical tumours, vulval tumours or OPSCC. Overall FANCD2 expression was significantly associated with sex of the patient in OPSCC with female sex having higher overall FANCD2 staining scores. There was no association between FANCD2 staining scores and smoking history or alcohol consumption in OPSCC dataset.

There was no significant association between overall, nuclear, or cytoplasmic FANDC2 scores with tumour recurrence in cervical or OPSCC cancer. In vulval cancer, lower nuclear FANCD2 scores was associated with an increased recurrence rate.

Survival analyses were performed using a Kaplan -Meier curve and cox-regression analysis. Neither overall, nuclear, or cytoplasmic FANCD2 levels were associated with disease-specific survival in the overall OPSCC cohort or when analysed by HPV status. HPV status and p16 status were significantly associated with disease-specific survival ($p<0.001$, $p=0.011$).

4.3.7 Discussion

This study suggests a differing effect on FANCD2 levels during malignant transformation of cervical cancer and vulval cancer. Cervical cancer is associated with an increase in FANCD2 nuclear expression when compared to normal cervical tissue, and this is supported in other recent publications which demonstrate a high rate of FANCD2 nuclear positivity in cervical cancers, and increasing FANCD2 levels during transformation from normal cervix to high grade CIN (162, 163). In contrast vulval cancers demonstrate a significant loss of FANCD2 nuclear expression when compared to normal tissues. A direct comparison of OPSCC tumour FANCD2 nuclear staining with normal oral mucosa was not possible due to limitation on available normal tissue, but previous studies have indicated a loss of nuclear FANCD2 staining in HPV positive OPSCC cancers (154), similar to that seen in vulval cancers. More recent publications have corroborated the finding that malignant transformation in OPSCC is characterised by a loss of FANCD2 nuclear staining (164). This would be consistent with the finding that FANCD2 staining in the OPSCC cohort was predominantly cytoplasmic.

Interestingly, both gynaecological tumours groups demonstrate an increase in cytoplasmic FANCD2 staining during malignant transformation. The roles of cytoplasmic FANCD2 are yet to be fully elucidated with immunoprecipitation studies demonstrating cytoplasmic FANCD2 interaction with members of the innate immune system (161). Increased cytoplasmic FANCD2 is associated with reduced expression of metastasis-associated proteins and improved outcomes in breast cancer, and also with improved survival in ovarian cancer (160). This study failed to demonstrate any association between cytoplasmic FANCD2 and survival in OPSCC. It is plausible that improved survival in tumours with high intensity of cytoplasmic staining could be due to loss of nuclear translocation of FANCD2

due to impaired DDR. A previous study found that cervical cancers with higher FANCD2 nuclear staining are associated with resistance to chemoradiation therapy (17).

Reasons for the loss of nuclear FANCD2/shift of FANCD2 to the cellular compartment in vulval (and probably OPSCC) tumours are unclear. Vulval cancer has been associated with loss of chromosome 3p25 (165), a potential mechanism for low FANCD2 expression. Cervical cancer appears distinct from the other two sites in that nuclear FANCD2 levels increase in malignant tissue. The FANCD2 antibody used identifies both native and ubiquitinated FANCD2, and therefore the distribution of activated FANCD2 in these tumours is not known. Further studies to identify the distribution of activated forms of FANCD2 would be desirable, and this could be done with antibodies recognising phosphorylated FANCD2 or by analysing FANCD2 nuclear foci formation by IF. This may also give further insight into any relationship between HPV status and FA pathway activation. The data does not support a role for HPV status influencing FANCD2 protein levels or subcellular localisation. A recent publication by Konos et al describes increased FANCD2 foci formation in HPV positive OPSCC when compared to HPV negative OPSCC (166), which was not apparent in this study looking at levels of all forms of FANCD2. Other work suggests preferential recruitment of FANCD2 to viral episome sites and impairment of FANCD2 localisation to cellular DNA damage (167, 168), and it would be of interest to look at FANCD2 foci formation dynamics post ionising radiation in HPV positive primary cells to determine if there was evidence of delayed repair kinetics in a model more representative of tumour tissue.

In this dataset, there was a suggestion that loss of nuclear FANCD2 in vulval cancers could be associated with an increased recurrence rate. If HPV negative OPSCC was analysed independently, there was a trend towards worse survival with low nuclear FANCD2 scores ($p = 0.07$). A recent publication in anal cancer (which is also largely HPV-associated) has demonstrated FANCD2 loss as a feature of metastatic or recurrent disease (169). Therefore, low nuclear FANCD2 could be a negative prognostic factor in vulval cancer/OPSCC but further work would be needed to establish if this were the case.

Chapter 5 – Transcriptomic Analysis of Cervical and Vulval tumours

5.1 Background

Immunohistochemical analysis of DDR protein expression has many advantages including the ability to analyse the functional protein levels, subcellular localisation and post-translational modifications reflecting protein activation such as phosphorylation. However, studies of this nature are limited to a small number of proteins due to the inability to multiplex assays and the time and tissue constraints associated with this. DDR proteins exist within complex cellular pathways, and therefore it is desirable to look at pathway function as a whole by analysing a wide range of DNA repair proteins in an unbiased approach. Taking this into consideration, an analysis of mRNA expression of cervical and vulval tumours was assessed utilising the Nanostring nCounter analysis platform to perform multiplex RNA hybridisation and quantification. When available, fresh frozen tissue samples were used in preference to FFPE tissue for RNA extraction to increase the quality of RNA although Nanostring technology was selected as it is recognised to yield results from lesser-quality mRNA extracted from archival FFPE samples. The PanCancer Pathway pre-defined codeset of 770 genes was chosen as a cost-effective means of assessing a wide range of mRNA probes relating to key DNA repair genes (including ATM, full list in Appendix C), whilst including multiple other cancer related genes for exploratory analysis.

The main aim of this analysis was to allow the identification of over or under-expression of DNA repair genes in gynaecological HPV-mediated cancers. A secondary objective was to perform a broader analysis of the expression of genes within the PanCancer codeset, a group of genes selected on the basis of an association with oncogenesis. There is a paucity of published data regarding the transcriptomic profile of vulval carcinomas, and this approach would generate novel data in this field. As this work was planned, a large TCGA dataset of 178 tumous analysed on the Illumina HiSeq platform was published as part of comprehensive molecular overview of cervical cancer in 2017 (101). The samples analysed were comparable to the smaller cohort included in this study, mostly of early stage disease and HPV positive. In this publication the cervical cancers demonstrated molecular heterogenity when analysed on the iCluster20 software, based on the 500 most variable gene copy number, methylation. mRNA and miRNA expression. Three molecular clusters were identified (keratin-low, keratin-high and adenocarcinomas), largely corresponding to

the mRNA expression clusters (C3, C2 and C1) ,based on 300 signature genes (Appendix C). This study highlights the potential utility for mRNA analysis as a means of molecularly characterising cervical cancers in a more resource-limited setting. This study therefore aimed to explore if the commercially available nanostring PanCancer codeset can segregate cervical tumours into potentially clinically meaningful categories based on a limited mRNA profile which can be performed rapidly and relatively inexpensively.

5.2 – mRNA Extraction and Sample and Data Quality Control

Tumour and cervical normal tissue FFPE blocks were obtained and evaluated by H&E staining (as described previously in Chapter 4). 1mm cores of tumour or normal tissue were obtained using the Alphametrix MTA-1 manual arrayer. In addition, fresh frozen tissue samples were available from the study 'Tumour tissue procurement to allow establishment of primary cell cultures for use in Human papillomavirus-mediated cancer research' (protocol version 1.2, Appendix A). Once thawed, the frozen tissue samples were dissected into small pieces with a sterile scalpel and trimmed to remove any macroscopic areas not containing epithelium such as blood vessels. Frozen tumour biopsies were taken only from areas of macroscopic identifiable tumour away from the clinical margin. Frozen samples were only included in the study if subsequent histological evaluation from fixed tissue removed concurrently or subsequently confirmed malignancy.

mRNA was extracted from a panel of FFPE and fresh frozen tissue using the Qiagen miRNAeasy kit as per manufacturer's protocol. The tissue was homogenised prior to mRNA extraction using the Precellys 24 system. The quantity and purity of the mRNA input was first assessed by Nanodrop spectrophotometer assessment. Extracted mRNA was then evaluated on the Agilent RNA 6000 bioanalyser for an assessment of the level of degradation of the RNA as quantified by the RIN (RNA Integrity Number), varying form 10 – 1, with the higher the number the more intact the RNA. Varying quality of mRNA by these metrics was reflective of biological sample of origin, with better quality demonstrated from fresh tissue (Table 5.1). Due to logistical constraints, the quality of RNA extracted from sample 30 was not analysed (the data from this sample was excluded from analysis based on data quality control). 5005N demonstrated poor quality of RNA and was excluded from differential expression analyses between tumour and normal tissue. A minimum of 100ng of mRNA was used for input into the experiment.

Data quality control was performed on the Nanostring data output using the n solver 4.0 software as per manufacturers guidance. No QC flags were raised, and all independent

parameters were within acceptable ranges. Background correction was not performed as the intention was to investigate fold changes and not to specifically identify for targets of interest with low levels of expression. True counts for most mRNA targets will far outnumber false positives as advised by manufacturer, thus the effect of the latter on fold-change estimates is anticipated to be negligible.

The probe count data was normalised by positive control normalisation and codeset content/housekeeper gene normalisation in the n solver 4.0 software. Normalised probe counts were expressed as a z score describing the position of the score in terms of its distance from the mean, when measured in standard deviation units. All housekeeping genes were critically examined to exclude any targets with notable instability in expression, and those with low counts (<100) were excluded. Two samples had mRNA content normalisation flags (samples 30 and 33), and sample 30 was excluded from analysis. Sample 33 was retained in the analysis as a minimum of 4 normal cervix controls were required to act as a baseline for differential expression analysis. Sample 33 had a content normalisation factor of 10.11 (very close to the recommended upper limit cut off of 10 as per manufacturers guidance).

All analysis was performed on the nsolver 4.0 and advanced analysis 2.0 software provided by Nanostring.

5.3 Tumour HPV Testing

All samples except sample 2 had HPV status determined by either p16 immunohistochemistry/RNA ISH on fixed tissue, and/or by RT-PCR for HPV 16 and HPV 18 E6 on mRNA extracted from fresh tissue. HPV testing and status is listed in Table 5.2. As discussed in Chapter 4, it was noted that a higher proportion of cervical cancer samples were labelled HPV negative by HPV RNA ISH and HPV 16/18 E6 RT-PCR than would be expected given the acknowledged prevalence in the general population. The TCGA dataset for cervical cancers demonstrated 9 out of 178 (5%) were HPV negative based on a consensus of MassArray and RNA-seq testing. In my study, 5 of the 17 cases of cervical cancer analysed (29%) were labelled HPV negative. The same limitations of HPV RNA ISH on the FFPE samples could apply as discussed in Chapter 4. P16 is recognised as a surrogate marker for HR-HPV in cervical tissue (170), and two samples (sample 7/5022T, sample 17/C10) were p16 positive in the absence of HPV RNA ISH positivity. For the purposes of this analysis these samples were considered positive for HPV based on their positive staining for p16. Sample 16/C8 was evaluated in three tumour cores as being negative for

p16, and HR-HPV RNA ISH and therefore is conclusively HPV negative, consistent with a small group of cervical tumours which are HPV inactive (171).

The cohort of fresh tissue tested by RT-PCR for HPV16/18 E6 would have benefitted from testing for a wider panel of HR-HPV subtypes. This is demonstrated by sample 5 where both FFPE and fresh tissue from the same tumour were available. This sample demonstrated positive p16 and HR-HPV RNA ISH positivity, but negative RT-PCR for HPV16/18 E6 which is suggestive of the presence of a different HR-HPV strain (HPV - 31, 33, 35, 52 and 58 also incorporated in the ACDBio RNAScope probe utilised for RNA ISH). The MassArray and RNA-seq utilised by the TCGA identified less common HPV strains (including 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 73). Sample 4 could have benefitted from wider testing for more HR-HPV subtypes if this had been available, and in the absence of p16/HR-HPV RNA ISH data its HPV status was determined as unknown for the purposes of this analysis.

5 out of the 7 vulval cancer samples were HPV negative, which is consistent with the lower prevalence of HPV-mediated vulval cancer, with HR-HPV implicated in approximately 43% cases (172). All 5 negative samples tested p16 negative.

Table 5.1 Summary of mRNA input into the Nanostring analysis from cervical/vulval tumours and normal cervix

Sample ID	Name	Histology	Sample type	Stage	Nanodrop 260/280	Nanodrop 260/230	RIN
1	5004T	Squamous cervix	Fresh frozen	IIB	2.06	2.24	8.3
2	5010T	Squamous cervix	Fresh frozen	IIIB	2.07	2.03	9.3
3	5011T	Squamous cervix	Fresh frozen	IIB	2.07	2.1	7.9
4	5013T	Squamous cervix	Fresh frozen	IB	2.08	1.6	8.3
5	5019T	Squamous cervix	FFPE	IIB	1.96	2.07	2.3
6	5020T	Adenocarcinoma cervix	FFPE	IB	1.92	1.82	2.2
7	5022T	Squamous cervix	FFPE	IIIC	1.97	2.07	2.4
9	C11	Squamous cervix	FFPE	IIB	1.97	2.12	3.8
10	C7	Adenocarcinoma cervix	FFPE	IIA	1.97	2.13	2.4
11	5005N	Normal cervix	Fresh frozen	n/a	1.84	0.45	no data
13	5016T	Squamous vulval	FFPE	IIIB	1.94	2.09	3.4
14	5026T	Squamous cervix	FFPE	IIIB	1.91	1.69	1
16	C8	Squamous cervix	FFPE	IB	1.9	1.83	1.4
17	C10	Adenocarcinoma cervix	FFPE	IB	1.86	2.01	2.3
18	V1	Squamous vulval	FFPE	IB	1.94	2.15	2
19	V9	Squamous vulval	FFPE	IB	1.97	2.23	2.2
20	5023T	Adenocarcinoma cervix	FFPE	IB	1.83	1.98	2.3
21	5015T	Squamous cervix	FFPE	IB	1.97	2.07	2.4
22	C3	Squamous cervix	FFPE	IB	1.97	1.89	2.2
23	NC10	Normal cervix	FFPE	n/a	1.93	1.75	2.3
24	NC19	Normal cervix	FFPE	n/a	1.94	1.86	2.4
25	5009T	Squamous vulval	Fresh frozen	II	*2.14*	1.99	3.5
26	5005T	Squamous cervix	Fresh frozen	IB	*2.11*	1.8	9.6
30	WRTB14-001	Squamous cervix	Fresh frozen	IIIB	no data	no data	no data
31	5002T	Squamous cervix	Fresh frozen	IB	2.06	2.17	3.3
32	NC20	Normal cervix	FFPE	n/a	1.96	2.03	2.4
33	NC23	Normal cervix	FFPE	n/a	1.97	1.78	2.3
34	5007T	Squamous vulval	Fresh frozen	IIIA	2.02	2	9.9
35	5008T	Squamous vulval	Fresh frozen	recurrence	2.06	2.23	9.5
36	5024T	Squamous vulval	FFPE	recurrence	1.88	1.46	1

Nanodrop 260/280 – ratio for determining purity of sample by assessing wavelength absorbance of contaminants. Pure RNA should be 2.0.

Nanodrop 260/230 – ratio for determining purity of sample by assessing wavelength absorbance of contaminants. Pure RNA should be 2.0-2.2.

RIN – RNA integrity Number as analysed on the Agilent RNA 6000 bioanalyser. Range 1-10 (higher number in this range represents superior RNA quality).

Table 5.2 Summary of HPV testing in cervical/vulval tumours and normal cervix samples prior to Nanostring analysis

Sample ID	Name	Tissue of origin	P16 status	HPV RNA status	HPV 16 E6 RT-PCR	HPV 18 E6 RT-PCR	HPV status
1	5004T	cervix	uk	uk	+	-	positive
2	5010T	cervix	uk	uk	uk	uk	uk
3	5011T	cervix	uk	uk	+	-	positive
4	5013T	cervix	uk	uk	-	-	uk
5	5019T	cervix	+	+	-	-	positive
6	5020T	cervix	+	+	uk	uk	positive
7	5022T	cervix	+	-	uk	uk	positive
9	C11	cervix	+	+	uk	uk	positive
10	C7	cervix	+	+	uk	uk	positive
11	5005N	cervix	uk	uk	-	-	negative
13	5016T	vulval	-	-	uk	uk	negative
14	5026T	cervix	-	-	uk	uk	negative
16	C8	cervix	-	-	uk	uk	negative
17	C10	cervix	+	-	uk	uk	positive
18	V1	vulval	+	+	uk	uk	positive
19	V9	vulval	+	+	uk	uk	positive
20	5023T	cervix	+	+	uk	uk	positive
21	5015T	cervix	+	+	uk	uk	positive
22	C3	cervix	+	+	uk	uk	positive
23	NC10	normal cervix	-	-	uk	uk	negative
24	NC19	normal cervix	-	-	uk	uk	negative
25	5009T	vulval	-	-	-	-	negative
26	5005T	cervix	+	+	-	+	positive
31	5002T	cervix	+	+	+	-	positive
32	NC20	normal cervix	-	-	uk	uk	negative
33	NC23	normal cervix	-	-	uk	uk	negative
34	5007T	vulval	-	-	uk	uk	negative
35	5008T	vulval	-	-	uk	uk	negative
36	5024T	vulval	-	-	uk	uk	negative

ISH = in situ hybridisation, RT-PCR = reverse transcription PCR, uk = unknown

5.4 Overview Analysis of PanCancer Pathway Codeset

Data from 29 samples was available for analysis of mRNA expression in cancer-associated pathway gene mRNA in cervical tumours (n=17), vulval tumours (n=7) and normal cervix tissue (n=5). As expected, unsupervised hierarchical clustering of the tissues sample demonstrated clustering of the normal cervix/adjacent normal cervix (Figure 5.1). There is a group of tumours that cluster with, but are distinct from, the normal tissue – this includes both of the HPV positive vulval cancers; sample 16/C8 (HPV negative, p16 negative cervical cancer); and 2 out of the 4 cervical adenocarcinomas included in the dataset. It is most likely that this is a result of different transcriptomic profiles of these tissues when compared to HPV positive squamous cervical cancer and HPV negative vulval cancer; although interpretation of this is made difficult by the small numbers of each tumour subtype. Another possibility is that the input tissue was less differentiated from normal tissue (possibly dysplasia) or was subject to a sampling error that resulted in the inclusion of adjacent normal tissue in the tissue samples from which mRNA was extracted. This would perhaps be less likely since tumour sampling was guided by H&E stained sections marked by a consultant histopathologist.

The cluster group to the right of Figure 5.1 contains predominantly squamous cancers. At this high level overview there does not appear to be any clustering of squamous tumours according to stage, HPV status, p16 status or anatomical (cervix, vulval) location. This data is also presented as Principal Component Analyses in Figure 5.2. Principal Component 1 (with the maximal amount of variance between the samples) demonstrates a trend towards seperation by tissue histology and HPV status. Adenocarcinomas appear to have a transcriptomic profile less differentiated from normal cervix and there is a trend towards some separation of cervical and vulval cancers, and seperation of HPV positive and negative tumours. With most vulval tumours being HPV negative and most cervical cancers with confirmed status being positive it is difficult to know if histological subtype of HPV status is the differentiating factor behind the PCA plot seperation.

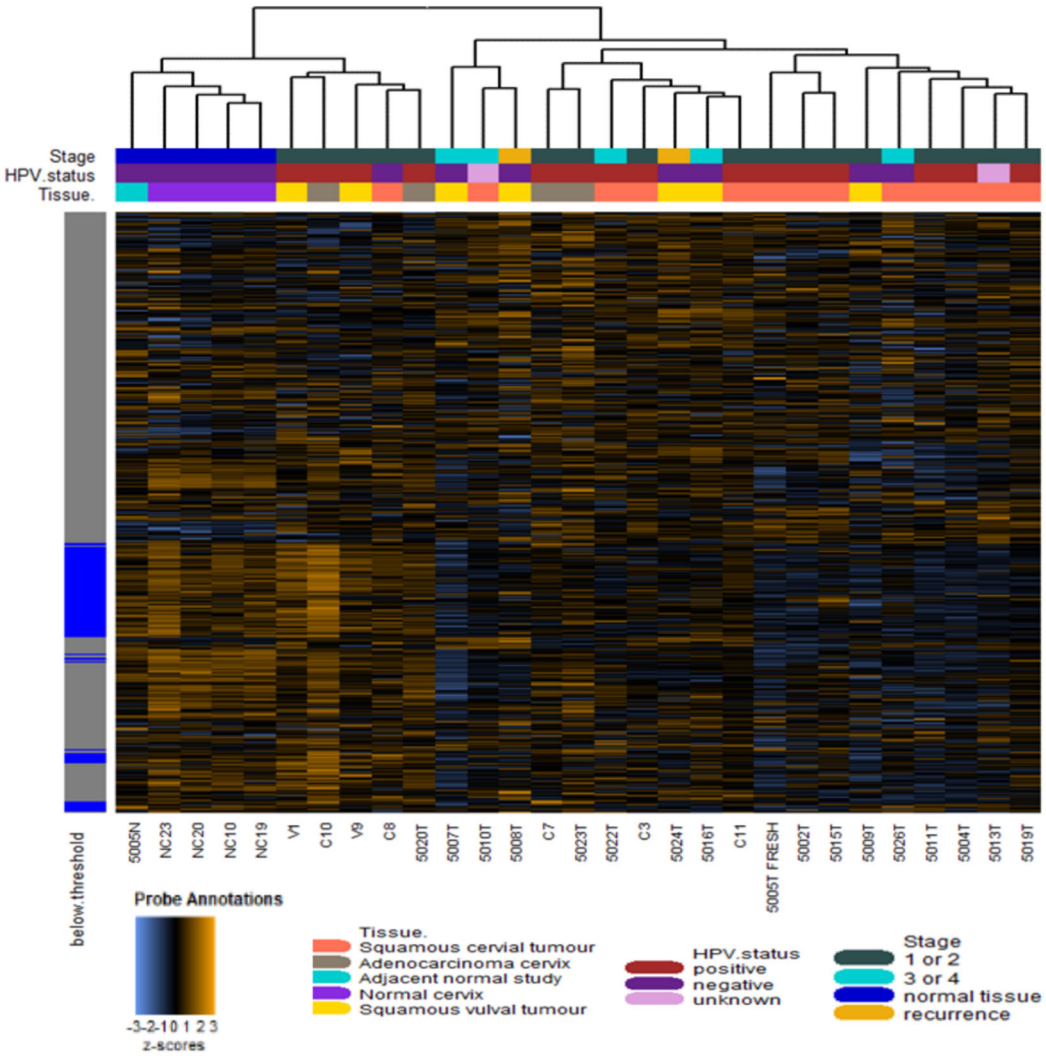


Figure 5.1 Heatmap demonstrating unsupervised hierarchical clustering of tumour and normal normalised probe counts expressed by a z-score value describing the distance of the value from the mean when measured in standard deviation units

z-scores measured -3 to 3 with blue representing low expression, black representing average expression and orange representing high expression.

Probes with below threshold counts for all samples (<25) shown as blue in column on the left side of the heatmap, and these probes will be excluded from further analyses (n=150)

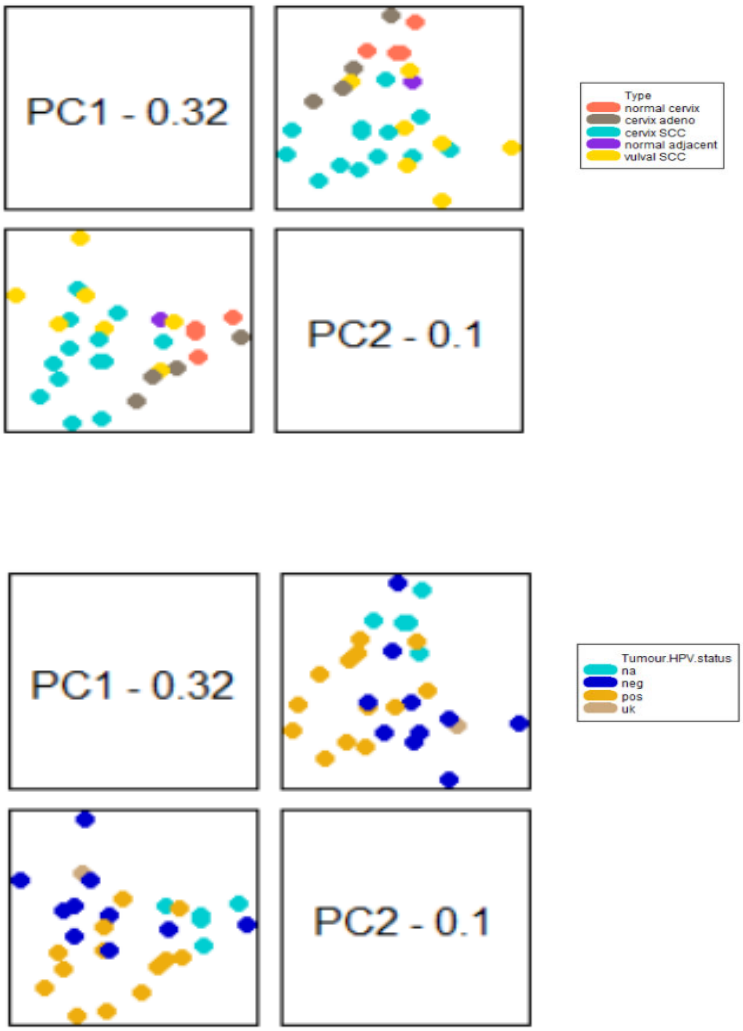


Figure 5.2 Principal Component Analysis of all samples according with samples labelled by Histological subtype (Type) and HPV status (Tumour hHPV status)

5.5 Analysis of Dysregulated Gene Expression in Cervical and Vulval Tumours

Differential expression analysis of genes was performed on all tumour samples with normal cervix as the comparator (due to the absence of normal vulval controls, which are difficult to obtain due to lack of routine surgical procedures removing normal vulval tissue). All probes with a log2fold change >3 (with an adjusted p value <0.05) in differential expression between any of the subgroups (squamous cervical cancer, vulval cancer, cervical adenocarcinoma) and normal cervix were identified. Many of the significantly over-expressed mRNAs were common to all the tumour subtypes studied (Table 5.3). The over-expression of these mRNAs may be universally implicated in the development of malignancy in cervical and vulval cancers.

There are a number of over-expressed mRNAs found in squamous cancers (both cervical and vulval), but not in cervical adenocarcinomas (*GZMB, MMP9, PLAU, IL12RB2, ITGA3, SPP1*). There is also a subset of mRNAs that appear to be highly expressed only in vulval cancers (*IL1A, IL1B, INHBA, FST, IL-8, IL-24, LAMA3, LAMB3, LAMC2*). The changes seen predominantly in the vulval tumours may be reflective of the different tissue of origin compared with control tissue (no normal vulval tissue as a comparator), but perhaps more likely reflecting the different pathogenesis of a predominantly HPV negative set of vulval cancer which may have arisen on the background of chronic inflammation (lichen sclerosis). In support of this hypothesis, IL 1 is a critical mediator of inflammation-associated carcinogenesis.

Many of the significantly under-expressed mRNAs identified were again common to both cervical and vulval squamous tumours, but not identified in cervical adenocarcinomas (Table 5.4). There is a subset of changes specific to vulval tumours, particularly involved with transcriptional misregulation (*HOXA11, PBX1, TMPRSS2*).

Table 5.3 mRNA probes demonstrating over-expression (log2foldchange >3) compared to normal cervix controls

Gene	All tumour expression log2 fold change (n=24)	Squamous cervical cancer expression log2 fold change (n=13)	Vulval squamous cancer expression log2 fold change (n=7)	Cervical adenocarcinoma expression log2 fold change (n=4)	Gene ontology
CCNE1	3.52*	3.55*	2.97*	4.09*	Cell Cycle - Apoptosis, PI3K
E2F1	3.79*	3.96*	3.03*	4.21*	Cell Cycle - Apoptosis
MCM2	2.39*	2.49*	1.53	3.05*	Cell Cycle - Apoptosis
IL1A	3.23	3.23	5.37*	0.118	Cell Cycle - Apoptosis, MAPK
IL1B	3.05	1.69	4.42*	0.654	Cell Cycle - Apoptosis, MAPK
BRCA2	2.97*	3.08*	2.78*	2.9*	DNA Repair
RAD51	5.27*	5.56*	4.78*	4.92*	DNA repair
FEN1	3.95*	4.12*	3.81*	3.55*	DNA Repair
EZH2	2.59*	2.52*	2.39*	3.05*	Driver Gene
IL12RB2	4.39*	4.2*	5.17*	1.72	JAK-STAT
IL24	1.34	1.43	5.17*	1.39	JAK-STAT
ITGA3	2.83*	2.88*	3.22*	1.36	PI3K
LAMA3	2.68	2.07	3.76*	0.248	PI3K
LAMB3	2.66	2.54	3.18*	1.64	PI3K
LAMC2	4.31*	3.57*	5.44*	2.06	PI3K
SPP1	3.92*	4.47*	3.05	2.02	PI3K
INHBA	5.34*	4.65*	6.27*	4.73*	TGF-beta
FST	4.14*	3.46	5.27*	1.2	TGF-beta
GZMB	2.61	2.65*	3.04*	0.885	Transcriptional Misregulation
HIST1H3G	2.95*	3.07*	2.83*	2.75*	Transcriptional Misregulation
IL8	2.1	1.31	5.61*	2.94	Transcriptional Misregulation
MMP9	4.12*	3.33*	5.28*	1.81	Transcriptional Misregulation
PLAU	3.12*	2.84*	3.94*	0.99	Transcriptional Misregulation

*Genes listed by gene ontology. * denotes statistical significance with adjusted p value <0.05*

Table 5.4 – mRNA probes demonstrating under-expression (log2foldchange >3) compared to normal cervix controls

Gene	All tumour expression log2 fold change (n=24)	Squamous cervical cancer expression log2 fold change (n=13)	Vulval squamous cancer expression log2 fold change (n=7)	Cervical adenocarcinoma expression log2 fold change (n=4)	Gene ontology
CDKN1C	-1.92	-1.82*	-3.23*	-1.05	Cell Cycle - Apoptosis
AR	-3.46*	-3.24*	-6.12*	-2.49	Driver Gene
FOXL2	-2.27	-2.44	-3.65*	-1.24	Driver Gene
GATA2	-2.85	-3.12*	-3.54*	-1.55	Driver Gene
PTCH1	-3*	-3.73*	-3.87*	-1.37	Driver Gene, Hedgehog
CNTFR	-3.9*	-4.18*	-3.37	-4.1	JAK-STAT
LIFR	-2.86*	-2.89*	-3.16*	-2.37	JAK-STAT
SOCS3	-2.32	-3.2*	-3.16*	-1.32	JAK-STAT
CACNA1D	-2.73	-3.62*	-4.8*	-0.866	MAPK
CACNA1H	-1.64	-1.85	-3.29*	-0.24	MAPK
MAPK10	-2.91*	-2.85*	-3.79*	-2.19	MAPK, Ras, Wnt
NR4A1	-3.55	-4.12*	-3.82*	0.731	MAPK, PI3K
SHC3	-3.59*	-4.36*	-3.76*	-2.1	Ras
COL24A1	-3.59	-3.22*	-4.64*	-3.08*	PI3K
IGF1	-3.07	-4.22*	-4.88*	-1.06	PI3K, Ras
ITGA8	-2.08	-2.65	-3.32*	-0.516	PI3K
LAMC3	-3.04*	-3.24*	-4.23*	-1.94	PI3K
PDGFD	-3.44*	-3.46*	-4.51*	-2.48	PI3K, Ras
EYA1	-2.91*	-4.08*	-2.44	-2.51	Transcriptional Misregulation
GRIA3	-2.85	-4.02*	-2.24	-1.94	Transcriptional Misregulation
HOXA11	-2.21	-2.44	-3.13*	-0.842	Transcriptional Misregulation
PBX1	-1.61	-1.3	-3.95*	-0.903	Transcriptional Misregulation
RUNX1T1	-2.67*	-3.24*	-3.05*	-1.36	Transcriptional Misregulation
RXRG	-1.86	-3.67*	-2.06	-0.489	Transcriptional Misregulation
TMPRSS2	-1.17	-1.14	-5.3*	0.19	Transcriptional Misregulation
DKK2	-2.07	-3.34*	-2.56	-0.737	Wnt
AXIN2	-2.39*	-2.44*	-3.17*	-1.51	Wnt
SFRP1	-2.32	-2.76	-4.75*	-0.601	Wnt
SFRP4	-5.75	-4.53*	-4.78*	-1.28	Wnt

Genes listed by gene ontology. * Denotes statistical significance with adjusted p value <0.05

Comparison between Tables 5.3. and 5.4 demonstrates that the most differentially expressed genes belong to a variety of different molecular pathways. To gain insight into which pathways demonstrate overall high levels of transcriptional dysregulation compared to normal tissue, the nsolver advanced analysis package was utilised to calculate pathway mRNA expression scores (Table 5.5). The scores are derived from the first Principal Components Analysis (PCA) scores (1st eigenvectors) for each sample based on the individual gene expression levels for all the measured genes within a specific pathway. Tumour tissues demonstrated a lower mean pathway score in all pathways except for DNA repair and cell cycle/apoptosis. This correlates with the gene ontology of highly overexpressed genes in Table 5.3. Cervical adenocarcinomas undergo less transcriptional dysregulation than the squamous tumours, with mean pathway scores closer to normal cervix.

Table 5.5 Mean pathway transcription scores by tissue type

	Normal cervix	Squamous cervical tumour	Adenocarcinoma cervix	Vulval cancer
Cell Cycle - Apoptosis	-6.23	1.80	-0.99	1.21
Chromatin Modification	2.28	-0.81	1.08	-0.54
DNA Damage - Repair	-3.58	1.10	1.4	-0.11
Driver Gene	5.52	-1.59	1.87	-1.56
Hedgehog	2.55	-0.88	0.88	-0.61
JAK-STAT	3.84	-1.57	1.73	-0.14
MAPK	5.70	-2.04	3.33	-1.39
Notch	2.38	-1.07	1.66	-0.39
PI3K	6.71	-2.30	3.99	-1.86
Ras	4.97	-1.51	2.51	-1.40
TGF-beta	2.82	-1.07	2.33	-0.95
Transcriptional Misregulation	4.99	-1.57	3.18	-2.08
Wnt	4.16	-1.37	2.33	-1.45

5.6 Molecular Profiling of Cervical and Vulval Cancers

5.6.1 All Cervical Cancers

To determine if the PanCancer Nanostring Codeset gene panel would demonstrate clustering of molecular subtypes of cervical cancers based on mRNA expression data, a separate unsupervised hierarchical clustering was applied to the cervical cancers samples only (Figure 5.3). This analysis highlights the distinct mRNA profile of cervical adenocarcinomas to the left of the heatmap. The cervical squamous cancer felt most likley to represent a HPV negative cervical tumour (sample 16/C8) also clusters away from the other cervical squamous cancers, demonstrating a molecular profile more similar to the adenocarcinomas.

Figure 5.3 demonstrates that the majority of cervical cancers are divided into two groups within the main cluster of samples– labelled Group A and Group B. Group A consists of cervical squamous cancers with two cases of adenocarcinomas. Group B is exclusively cervical squamous cancers. A comparison of the differential expression of individual mRNAs between the two groups of squamous cancers yields no statistically signifcant values when corrected for multiple testing, possibly as a consequence of the modest number of samples available in the analysis.

If the two groups of cervical cancers (A and B) are analysed by pathway expression, the PI3k pathway and transcriptional misregulation pathways have the most prominent difference between the two groups. This trend is also seen if Group A and Group B squamous tumours only are analysed (Figure 5.4).

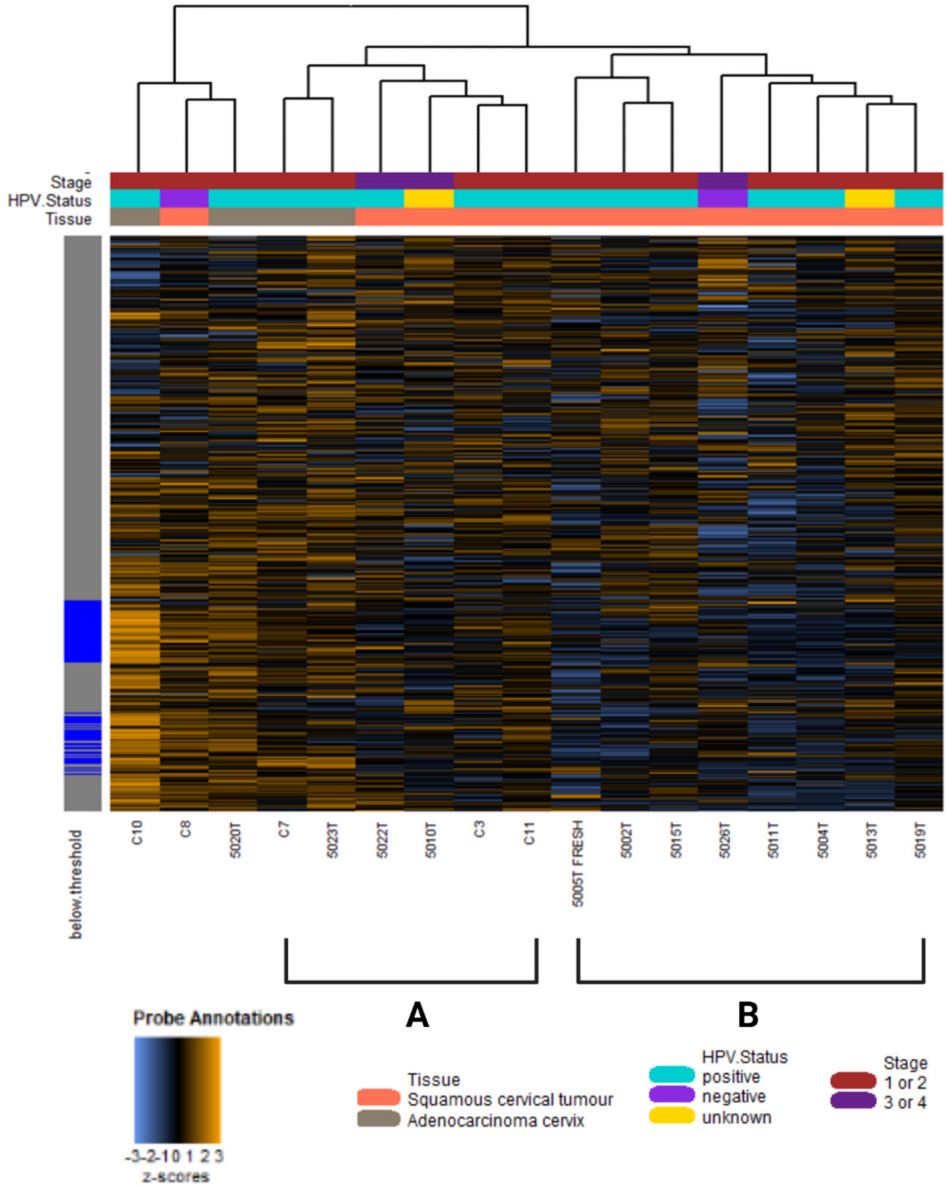


Figure 5.3 Heatmap demonstrating unsupervised hierarchical clustering of cervical tumour samples normalised probe counts expressed by a z-score value describing the distance of the value from the mean when measured in standard deviation units

z-scores measured -3 to 3 with blue representing low expression, black representing average expression and orange representing high expression

Probes with below threshold counts for all samples (<25) shown as blue in column on the left side of the heatmap, and these probes will be excluded from further analyses (n=150)

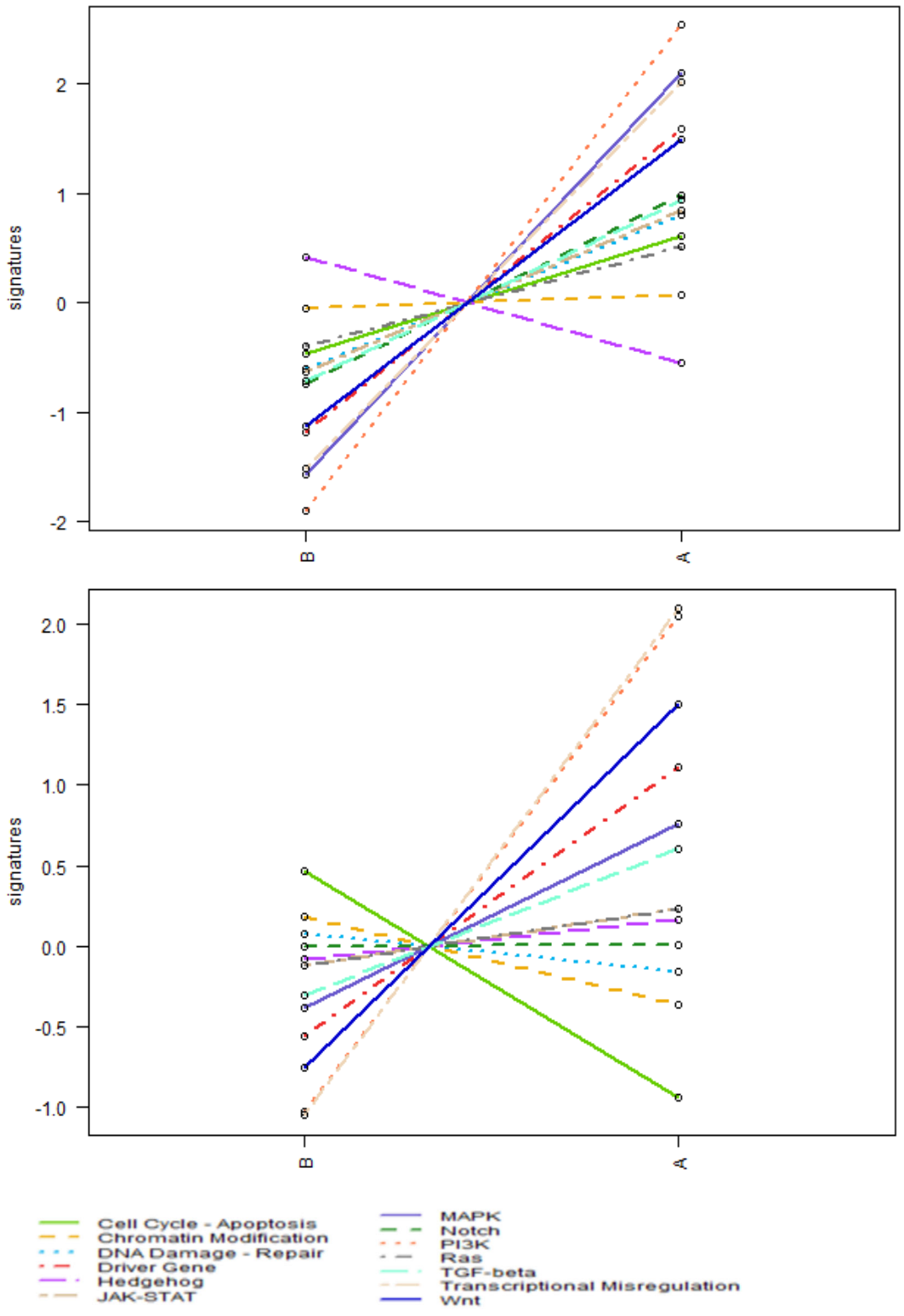


Figure 5.4 Diagram showing trends of mean pathway scores between Group B and A cervical Tumours

Top. All Group B vs Group A

Bottom. Squamous tumours Group B vs Group A

5.6.2 Squamous versus Adenocarinomas of the Cervix

A comparison of mRNA differential expression was also performed between cervical squamous and adenocarcinomas included in the study. As demonstrated in Figure 5.5, *NR4A1 and PLA2G1O* were both significantly over-expressed in adenocarcinomas when compared to squamous cancers (log2fold change 5.9, adjusted p value 0.045 and log2fold change 4.73, adjusted p value 0.012 respectively), even in this small sample of tumours (Figure 5.5). *NR4A1* is transcription factor implicated in mulitple cellular processes including activation of the mTOR pathway, TGF-β signalling, immune modulation, cell cycle progression and apoptosis and is assigned the MAPK, PI3kinase pathways on the nanostring codeset. It also plays a role in activation of apoptosis when translocated to mitochondria. *PLA2G10* is a member of the phospholipase A2 family of proteins associated cleavage of phospholipids, realeasing them from the cell membrane.

5.7– Analysis of Vulval Cancer mRNA Expression Profiles

HPV positive and HPV negative vulval tumours cluster separately on a heatmap demonstrating unsupervised clustering utilising z scores (Appendix C), but the numbers are too small to compare the differential expression between the two cohorts.

There are many observable similarities between the top differential expression of mRNA probes and mean pathway mRNA expression scores between squamous cervical and vulval tumours (Tables 5.3, 5.4 and Figure 5.6). However, the vulval cancer cohort demonstrated some features not common to cervical tumours, particularly in the increased expression of some inflammatory (*IL1A, IL1B, IL-8. 1L-24*) and basement membrane proteins (*LAMA3, LAMB2, LAMC2*) and the decreased expression of some members of the transcriptional misregulation pathway (*HOXA11, PBX1, TMPRSS2*). These findings were reflected in a direct comparison of differential expression between cervical and vulval tumours (Figure 5.6).

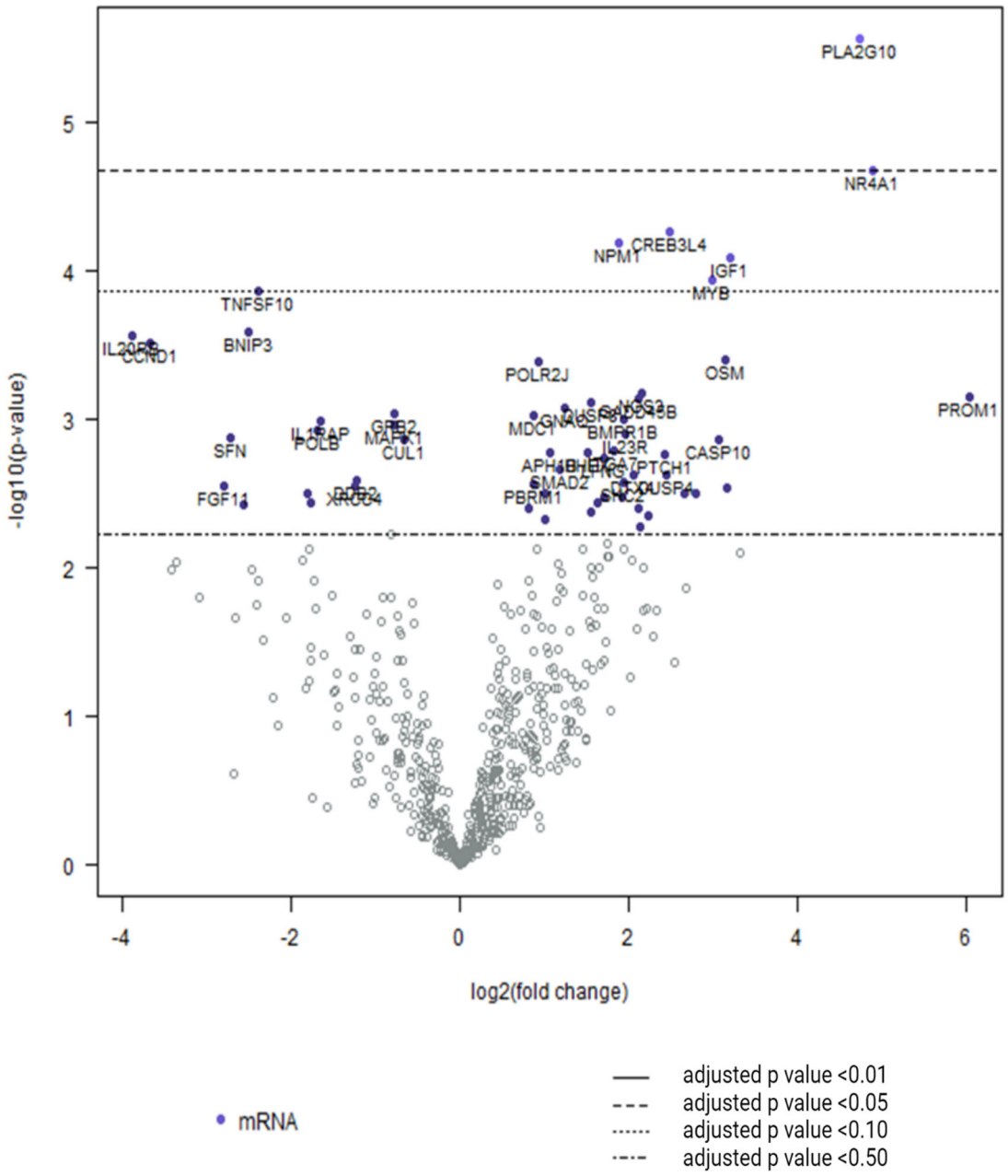


Figure 5.5 Volcano plot of log2 fold change in mRNA probe expression of cervical adenocarcinomas compared with cervical squamous cancers

p value of differential probe count corrected for multiple analyses and displayed on -log10 scale. mRNA probes with >0.50 p value are named

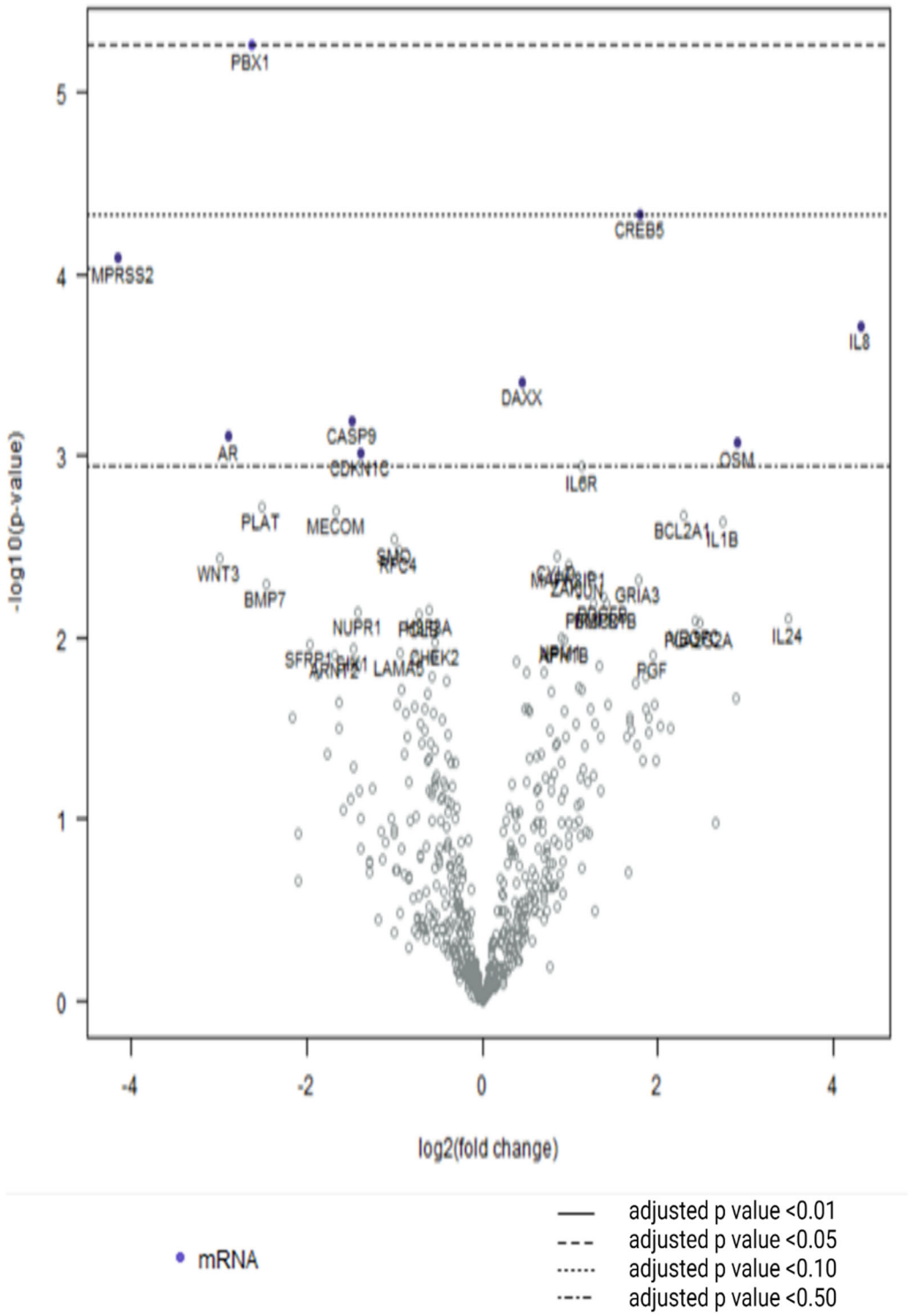


Figure 5.6 Differential expression of individual genes between vulval squamous cancers and cervical squamous cancers p value of differential probe count corrected for multiple analyses and displayed on -log10 scale.

5.8 HPV Positive versus HPV Negative Cancers

There are insufficient cases of HPV negative cervical cancers and HPV positive vulval cases to directly compare mRNA probe expression between HPV positive and HPV negative within the same anatomical location. A comparison between HPV positive and HPV negative tumours was performed in all squamous cancers with a confirmed HPV status. There were no significantly differently expressed genes between HPV positive and negative tumours if all tumour types are included. When analysing mean pathway scores, it can be appreciated that there are several pathways with significantly increased scores in HPV positive tumours when compared to HPV negative tumours. These include cell cycle-apoptosis, Ras, PI3kinase, transcriptional misregulation, driver genes, MAPK and DNA repair (Figure 5.7). Similar pathways (DNA repair, cell cycle-apoptosis, driver gene) are highlighted as having increased transcription when vulval tumours are analysed independently of cervical but PI3kinase, Hedgehog and TGF beta pathway scores are lower in HPV positive compared to negative in the vulval cohort.

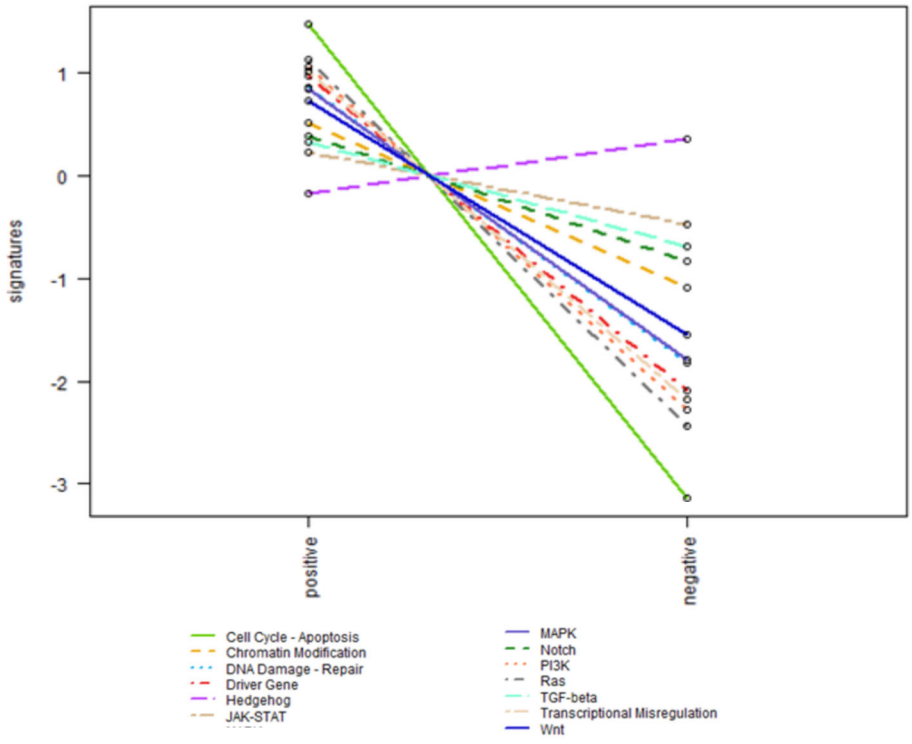


Figure 5.7 Diagram showing trends of means pathway scores between all HPV positive squmous tumours and all HPV negative squamous tumours

As a pathway of particular interest to this study, the volcano plot for the expression of DNA repair genes is shown in figure 5.8. This shows a global non-significant decrease in DNA repair gene expression in HPV negative tumours compared to HPV positive.

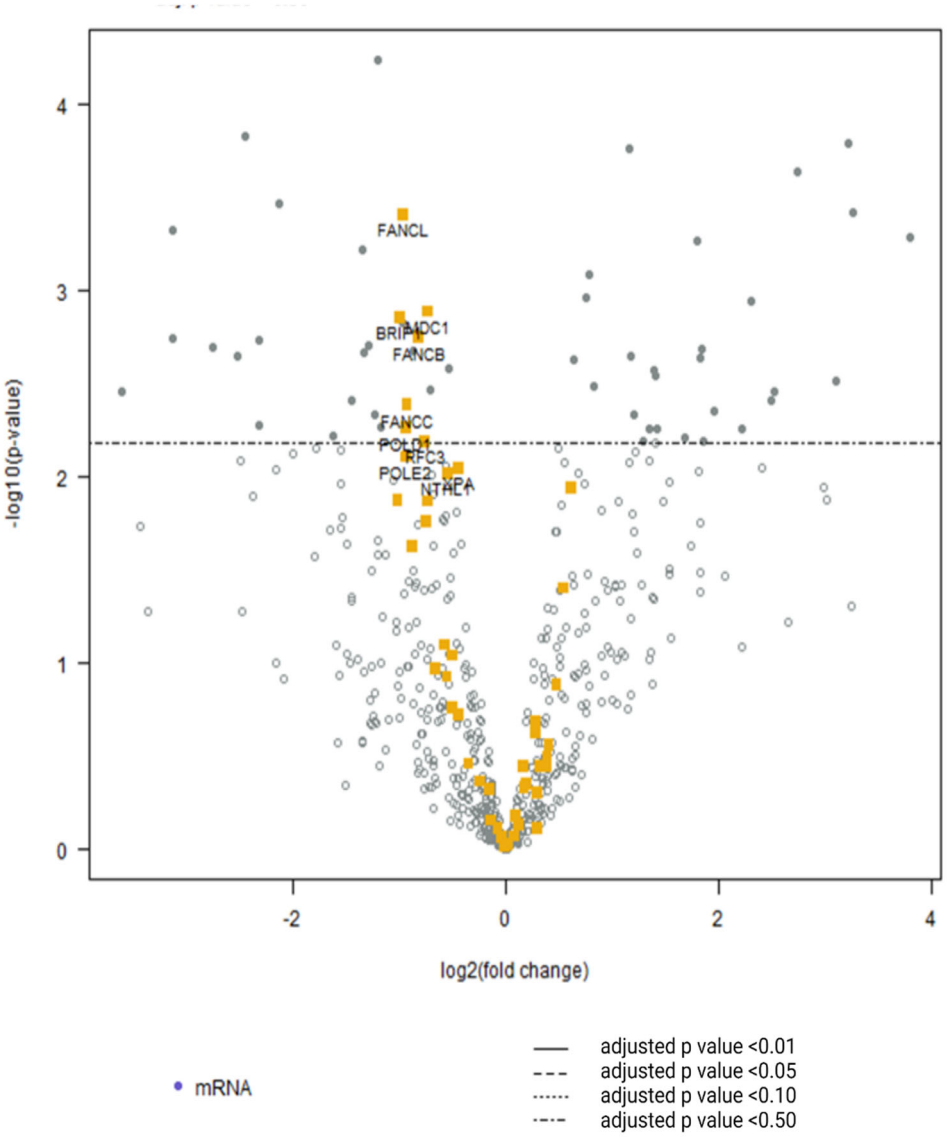


Figure 5.8 Differential expression of DNA repair genes (yellow squares) in HPV negative versus HPV positive cancers

5.9 Analysis of DNA Repair Pathway in Cervical and Vulval Tumours

5.9.1 – DNA Repair Pathway Signature in Cervical and Vulval Tumours

As a pathway of particular interest in this work, a more detailed assessment of the DNA repair codeset was performed; in particular with reference to the HRR/BRCA/FANC pathway.

Analysis of all DNA repair gene probe expression demonstrates clustering of the samples according to histological subtypes (normal, adenocarcinoma and squamous cancers) (Figure 5.9). As shown previously in this dataset, there is widespread upregulation of DNA repair genes in cervical and vulval cancers versus normal tissue, and this appears to be most prevalent in the HPV positive cervical squamous cell tumours to the right of the heatmap.

At this high level overview, it can be seen that there are individual samples with downregulation of single DNA repair genes compared to normal tissue and samples with low expression of DNA repair gene probes compared to other tumours. It is recognised that loss of activity of individual DNA repair genes can reduce HRR proficiency in tumour cells, and a closer look at the heterogeneity of mRNA expression within the HRR/BRCA/FANC mRNA probes was performed (Figure 5.10). There are 6 HRR/BRCA/FANC genes that are upregulated in all tumours when compared to normal cervical controls *RAD51, BRCA2, FANCA, BRIP1, UBE2T* and *BRCA1* (Table 5.6) and this can be clearly appreciated in Figure 5.10, particularly in the left hand cluster of tumours representing HPV positive cervical tumours. Figure 5.10 also demonstrates that certain probes such as *RAD50* and *ATM* appear to have globally less expression in tumours than normal tissues. The certain probes in the FA pathway have low expression in isolated samples including *FANCC, FANCF, FANCG, FANCE* and *c19orf40/FAAP24*. Of note the normal adjacent cervix sample (5005N) shows low transcription of *FANCB* and *FANCF* genes. Of note the RNA input was of poor quality for this sample but it would be interesting to confirm these findings as this may provide evidence of impaired FA pathway gene expression in cervical tissue from which a HPV 18 positive cancer arose.

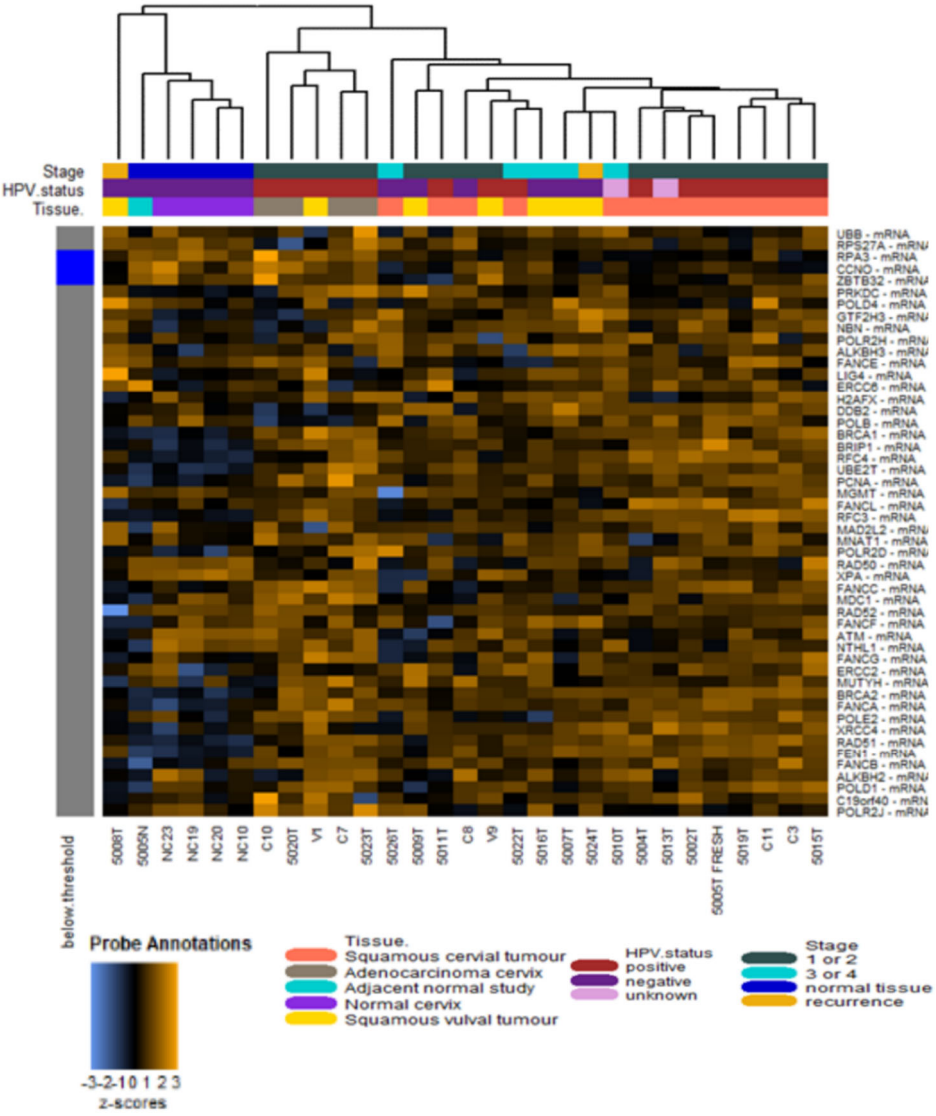


Figure 5.9 Heatmap demonstrating unsupervised hierarchical clustering of tumour and normal samples according to the normalised mRNA probe count of the DNA Repair genes within the Pan Cancer Pathway codeset

Sample normalised probe counts expressed by a z-score value describing the distance of the value from the mean when measured in standard deviation units

z-scores measured -3 to 3 with blue representing low expression, black representing average expression and orange representing high expression

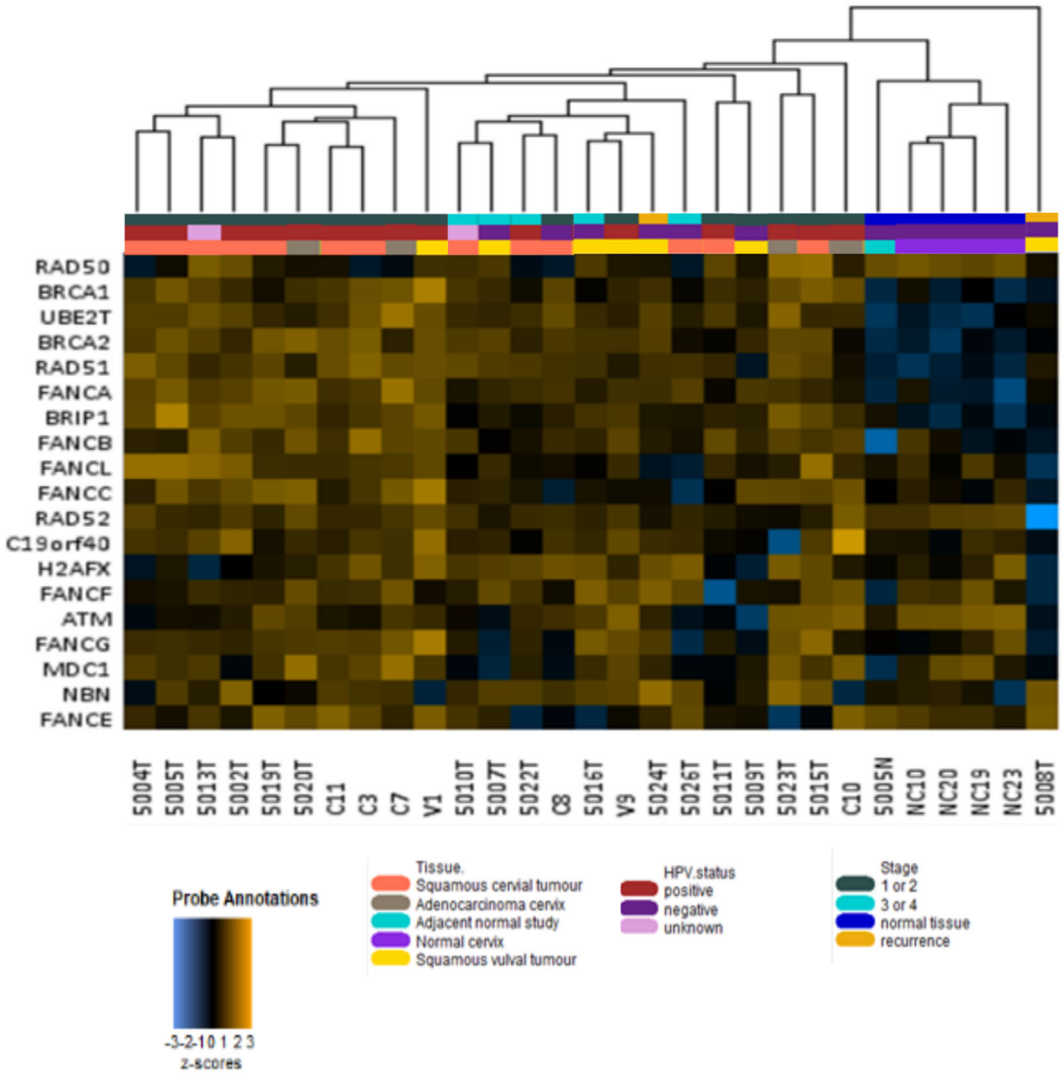


Figure 5.10 Heatmap demonstrating unsupervised hierarchical clustering of tumour and normal samples according to the normalised mRNA probe count of BRCA genes within the Pan Cancer Pathway codeset

Sample normalised probe counts expressed by a z-score value describing the distance of the value from the mean when measured in standard deviation units

z-scores measured -3 to 3 with blue representing low expression, black representing average expression and orange representing high expression

Table 5.6 mRNA probes from HRR/BRCA/FANC DNA repair pathways with statistically different ($p < 0.05$) expression between all tumours and normal cervix, in descending order of log2fold change of probe count

Probe Label	Log2 fold change (All tumours)	P value
RAD51	5.27	0.0003
BRCA2	2.97	0.0003
FANCA	2.56	0.0032
BRIP1	2.53	0.0007
UBE2T	2.43	0.0002
BRCA1	1.76	0.0081

5.9.2 Comparison of ATM mRNA probe expression levels and pATM protein expression on paired samples

Despite a global up-regulation of many key DDR genes, there is no increase in *ATM* expression seen, which is perhaps surprising given the findings in Chapter 3 demonstrating increased levels of pATM in cervical cancer. 10 cervical tumours with mRNA probe data also had pATM immunohistochemical staining scores and these were utilised to compare tumour ATM expression levels with pATM overall staining. There was a trend towards an inverse correlation between overall pATM staining and *ATM* transcriptional activity (Figure 5.11).

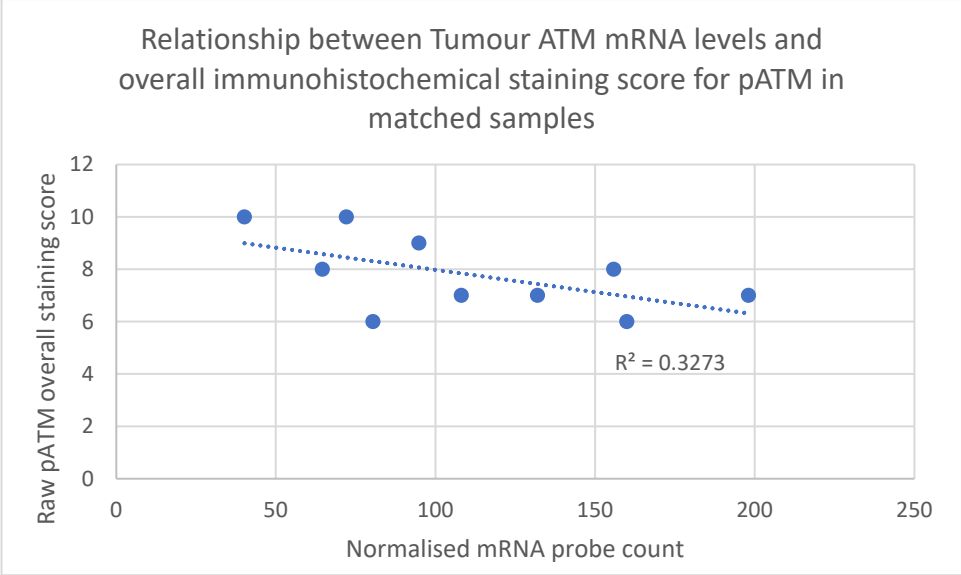


Figure 5.11 The Relationship between Tumour ATM mRNA levels and overall immunohistochemical staining score for pATM in matched samples

5.10 Discussion

5.10.1 Overview of Differential Gene Expression between Tumour and Normal Tissue

Analysis of a pre-defined set of mRNA probes selected for their role in pathways related to oncogenesis (Nanostring PanCancer codeset) demonstrates clear differences between mRNA expression profiles in tumour and normal samples, as may well be anticipated. Common features shared of all cancers when compared to normal tissue include high expression of genes promoting cellular proliferation including *cyclin E (CCNE1), E2F1, EZH2* and *MCM2* (Table 5.3), and low expression of *p57(CDKN1C*) whose function inhibit cell cycle progression (Table 5.4). A second common feature is high expression of key genes involved in HRR *RAD51, BRCA2,* and *FEN1*. In accordance with these findings cell cycle/apoptosis and DNA repair are the most dysregulated pathways in these tumours (Table 5.5).

5.10.2 Cervical Adenocarcinomas

Unsupervised hierarchical clustering of the mRNA expression data (Figure 5.1) and analysis of the mean pathway scores (Table 5.5) show that adenocarcinomas have lower levels of transcriptional dysregulation than the two squamous cancer groups when compared to

normal cervical tissues, despite an often shared aetiology (HPV infection). Squamous cervical cancers and adenocarcinomas significantly differ in their transcriptional profiles (Figure 5.5), with the most prominently dysregulated gene in cervical adenocarcinomas when compared to squamous tumours is the increased transcription of *PLA2G10*. PLA2G10 is a phospholipase A2 protein, with a role lipid metabolism, primarily functioning by cleaving extracellular phospholipids. This family of proteins are associated with cancer progression by releasing fatty acids into the tumour microenvironment promoting tumour growth, angiogenesis and invasiveness (173). Increased expression of *PLA2G10* has been associated with colorectal cancer and breast cancer (174, 175), but not previously reported in cervical adenocarcinomas. It could possibly represent a unique therapeutic target in adenocarcinoma of the cervix. All other significantly dysregulated mRNA probes in adenocarcinomas compared to normal cervix are shared with the other tumour groups and are pre-dominantly implicated in the cell-cycle/apoptosis/DNA repair pathways.

5.10.3 NR4A1 as a potential therapeutic target in Gynaecological Squamous cancers

A significant differentiating factor between cervical adenocarcinoma and the squamous tumours in the study was the significantly reduced expression of the orphan nuclear receptor *NR4A1* (also known as *Nur77*) (Figure 5.5, Table 5.4). This is perhaps surprising as NR4A1 is a transcription factor coded by an immediate early response gene in response to cellular stress, hypoxia and inflammatory and growth factors which are present in the tumour environment. No endogenous ligand for NR4A1 has been identified and therefore its activity is thought to be regulated via transcriptional control and cellular localisation.

NR4A1 nuclear transcription targets include genes promoting proliferation and angiogenesis, but conversely nuclear export of NR4A1 facilitates interaction with Bcl-2 in mitochondria promoting apoptosis. Loss of NR4A1 is of clinical interest as multiple studies in other tumour sites have demonstrated a key role for the NR4A1 in mediating platinum chemotherapy induced apoptosis. This may be of particular importance in cells with reduced function of p53, due to HPV activity or p53 mutation. Low levels of NR4A1 have been associated with chemotherapy resistance in high grade serous ovarian cancer (176), and NR4A1 has been identified as a therapeutic target to promote tumour cell apoptosis and overcome treatment resistance. Pre-clinical studies in Hela cells have demonstrated the potential to increase the translocation of NR4A1 with pharmacological means (177).

It is not clear what the mechanism is for reduced *NR4A1* transcription in cervical and vulval squamous cancers. Export of NR4A1 to the mitochondria is inhibited by PI3K/Akt activation and promoted by MAPK10/JNK3 signalling. The PI3Kinase/Akt pathways are thought to be upregulated in cervical and vulval cancers as 41% of cervical cancers have an activating PI3Kinase mutation in the TCGA study and 60% HPV positive vulval cancers have PIK3C activation (102). MAPK10 transcription is significantly down-regulated in this study(and corroborated by Zhang et al (178). It would therefore appear that the balance of NR4A1 nuclear export would be in the favour of nuclear retention based on regulating pathway activation. NR4A1 forms heterodimers with retinoic acid receptors to facilitate nuclear export and mitochondrial targeting. This study showed that Retinoic acid receptor gamma (RXRG) is significantly downregulated, again postulating another mechanism for nuclear retention of NR4A1. It is therefore possible that *NR4A1* transcription is reduced by negative feedback from high nuclear levels of the protein, however human protein atlas studies suggest that NR4A1 protein expression is low in cervical cancers. The regulation of N4A1 is complex and many factors have been associated with its induction including cytokines, growth factors, angiogenic factors and oxidative stress mediators, but of particular interest to this study, exogenous expression of HR-HPV E1 reduces NR4A1 expression, particularly in HPV 16 cancers, which may be beneficial to allow tolerance of cellular replication stress (179). This does not explain the differential expression between cervical squamous and adenocarcinomas, but in general adenocarcinomas are more often HPV 18 positive rather than HPV16 (180), although the HPV subtype of the adenocarcinomas in this cohort is unknown.

In conclusion, these finding suggest a low level of *NR4A1* expression in HPV-mediated gynaecological squamous cancers, the mechanism for which is unclear. Low NR4A1 levels and nuclear export may impair therapy-induced apoptosis with DNA damaging agents. Increasing NR4A1 nuclear export and enhancement of its pro-apoptotic effects in mitochondria could possibly enhance therapeutic efficacy and improve patient outcomes.

5.10.4 Mediators of Invasion in Gynaecological Squamous cell Cancer

A striking feature of the pattern of upregulated gene expression in the squamous cancer cohort was their association with cancer invasion. This study demonstrated upregulation of *Laminin 332* (previously known as *Laminin 5) gamma 2 chain (LAMC2), ITGA3, MMP-9* and Plasminogen Activator Urokinase (coded by *PLAU* gene) which confirmed previous findings in cervical and vulval squamous cancers (181-184). These genes are key effectors of tumour

invasiveness and are early events in the development of invasive squamous cancers (185, 186). The Laminin 332 gamma 2 chain has previously been identified as being highly expressed at tumour invasive fronts in oral squamous cancers and appears to be expressed in preference to the Laminin gamma 3 chain (LAMC3) which is significantly under expressed compared to normal tissue. Studies in laryngeal cancer have demonstrated the utility of cetuximab in inhibiting LAMC2 expression (187), although to date trials of cetuximab in cervical cancer have demonstrated disappointing efficacy. In vulva cancer *LAMA3* and *LAMB3* encoding Laminin 332 subunit alpha 3 and beta 3 were also significantly upregulated in this study.

In cervical squamous tumours the *SPP1*/osteopontin gene was over expressed which is thought to potentially contribute to cancer invasion and metastasis (188). NR4A1 inhibits osteopontin expression in the non-malignant setting (189). A recent publication analysing public datasets has corroborated the finding of increased osteopontin expression in cervical cancer and this was associated with activation of signalling of multiple pathways involved in cancer proliferation, angiogenesis and epithelial-mesenchymal transition (190).

5.10.5 Cytokine profile of Vulval Squamous Cancer

The analysis demonstrated some features specific to vulval squamous cancer transcriptomic profile, particularly with reference to significant increases in cytokines *IL1A*, *IL1B, IL8* and *IL24.* This is in keeping with the predominant aetiology in this cohort of vulval cancer being non-HPV and most probably due to a chronic inflammatory disorder such as lichen sclerosus, although HPV infection has also been shown to promote IL-1A expression (191, 192). These cytokines have recognised roles in cancer in the context of chronic inflammation promoting invasiveness and angiogenesis (193-195).

The consequence of increased levels of these cytokines on the tumour immune environment is intriguing as this may have consequences for immunotherapy treatment strategies in vulval cancer, such as pembrolizumab which have shown some promise in selected patients in the KEYNOTE 158 study (196). IL-1A has been shown to supress tumour immune surveillance and T cell activation when expressed at high levels, and IL-1B and IL-8 are both implicated in tumour infiltration of neutrophilic myeloid-derived suppressor cells (N-MDSC) which can promote immune escape. Elevated baseline IL-8 has been associated with poor responses to immune checkpoint inhibitor therapy in other tumour groups (197).

In contrast IL-24 is a tumour suppressor gene associated with promotion of a pro-inflammatory Th1-type response and inhibiting angiogenesis (198). IL-24 has been investigated as an adjunct to HPV E7 DNA vaccine therapy in cervical cancers to boost immunogenicity (199). The high level of expression of *IL-24* in the vulval tumour environment could be investigated as a biomarker to identify those who may benefit for immunotherapy (PD-L1 status was not predictive in the KEYNOTE 158 study). The *IL-12RB2* is also upregulated in cervical and vulval cancers, which could potentiate Th1-type inflammatory signalling via the IL-12 cytokine, and this has not been previously reported. IL-12 has been trialled therapeutically to promote anti-tumour immunity and techniques to develop local administration are promising to minimise systemic effects (200).

5.10.6 Predicted Pathway alterations in Cervical and Vulval Cancers

The mean pathway transcription scores generated by the Nanostring nsolver advanced analysis software (Table 5.5) give an overview assessment of pathway transcriptional dysregulation but gives little information with regards to the likely functional consequences and the potential impact of transcriptional changes on pathway activators and inhibitors. Table 5.7 summaries the key differentially expressed pathway genes identified in Tables 5.3 and 5.4 and their predicted effect on pathway status.

Loss of *PTCH1* has been previously identified in cervical and vulval squamous cancers and it has been recognised as an adverse prognostic feature (201-203). Wnt pathway activation has previously been linked to the development of cervical cancer, a process thought to be related to HPV E7/E7 presence (204). Activators of PI3kinase signalling are under and over expressed, and this may reflect constitutive pathway activation due to PI3kinase mutations described in the literature for cervical can vulval tumours.

Pathways that play a less clear role in cervical and vulval cancers based on the most significantly dysregulated genes are the jak-stat pathway, MAPK pathway and TGF-beta signalling. SOCS3 is a negative-feedback loop regulator of the jak-stat signalling pathway and therefore the low expression of this could potentially be a reflection of low levels of pathway activation, which would be consistent with the under-expression of the CNTF and LIF. Such a strategy may be useful in avoiding the anti-tumour action of jak-stat associated cytokines such as IL-24 and IL-12, which are elevated in vulval tumours as earlier discussed. Follistatin (FST) an inactivator of Activin A, likely reduces Activin A activation of TGF beta signalling in the squamous cancers studied. However, the INHBA gene coding an inhibin protein is widely over-expressed in all the tumours studied, including cervical

adenocarcinomas, and acts to activate TGFbeta signalling. This may counteract the increased presence of follistatin, and other proteins known to activate TGF beta signalling such as MMP9 also show high levels of transcription. Previous studies have demonstrated TGF-beta signalling loss during malignant transformation from CIN to invasive cancer (205). More in depth analysis of these pathways would be required to gain a comprehensive insight into signalling in these complex cases but given the scarcity of transcriptomic information available on vulval cancers in particular, these findings add to the current knowledge of signalling pathways.

Table 5.7 Summary of the most dysregulated genes belonging to oncogenic signalling pathways in cervical and vulval squamous cancers and predicted consequences of the changes in gene expression

Pathway	Upregulated Genes	Down regulated Gene	Predicted consequence of transcriptional dysregulation
Hedgehog		PTCH1	Pathway signalling activation
Wnt		DKK2, SFRP1, SFRP4, AXIN2	Pathway signalling activation
PI3kinase	SPP1/osteopontin, ITGA3, LAMA3, LAMB3, LAMC2	IGF-1, PDGFD, ITGA8, LAMC3, COL24A1	Unclear
RAS/MAPK		NR4A1, CACNA1D, CACNA1H, MAPK10, SHC3	Pathway signalling inhibition
JAK/STAT		CNTF, LIF, SOCS3 receptors	Pathway signalling inhibition
TGF-beta	FST, INHBA, MMP9		Unclear

5.10.7 Molecular sub-classifications of cervical cancer

The molecular profiling by the TCGA collaborative categorised cervical cancers into 3 groups based on mRNA expression (C1, C2 and C3), and this largely correlated to the three molecular clusters identified by the paper (adenocarcinomas, keratin-high and keratin-low)(101). The cervical cancers in this study can be divided into three groups; adenocarcinomas (including one HPV negative cervical cancer), 'Group A' (2 adenocarcinomas and squamous carcinomas) and 'Group B' (exclusively squamous carcinomas). These three groups correlate with the histological characteristics of the TCGA groups C1, C2 and C3. The TCGA C1 mRNA group contained adenocarcinomas and a group of squamous cancers which were termed endometrial-like cervical cancers (UCEC), which included 7 out of the 9 HPV negative cervical cancers. UCEC tumours had a molecular profile consistent with endometrial orgin but were histologically of cervical orgin. This raises the possibility that sample 16/C8 is a UCEC as described by TCGA analysis.

The TCGA C2 mRNA cluster was designated a 'keratin high' subgroup which consisted of mostly HPV positive squamous tumours characterised by high expression of members of the keratin family of proteins, whilst the C3 cluster was designated 'keratin low' and contained squamous cervical cancers and a subset of adenocarcinomas. Histologically these two groups correlate with Group B and Group A respectively. It appears that the nanostring PanCancer codeset mRNA profiles of the cervical tumours cluster cervical tumorus into similar histological groups as the more extensive TCGA analysis.

To determine if these groups correlate with the TCGA subgroups on a molecular level is more challenging (the nanosting codeset used does not contain keratin gene probes). Due to the small number of samples analysed, there are no statistically significantly different individual probes between the two predominantly squamous cervical cancer groups once corrected for mulitple testing. The TCGA dataset identified multiple significantly differentially expressed mRNA probes between the C2 and C3 mRNA expression clusters including *TGFB1*,*TGFB2, TGFBR2, PIK3CA, FGFR3, NFE2L2, EP300, MAPK1, KRAS, ARID1A* and *FBXW7* which are included in the nanostring codeset. These genes are predominantly involved in the TGFbeta signalling pathway (*TGFB1, TGFB2 and TGFBR2*), the PI3kinase pathway signal transduction (*PIK3CA, FGFR3*), RAS/MAPK pathway signal pathway (*KRAS, MAPK1, FGFR3*) and transcriptional misregulation (*NFE2L2, EP300, ARID1A*).

Figure 5.4 demonstrates the mean pathway score changes between Group A and Group B cervical tumours highlighting the PI3kinase, MAPK and transcriptional misregulation

pathways as being differentially expressed between the two groups.The TGF beta pathway does not show a large degree of differentation in pathway scores.

A futher anaylsis was done via the advanced analysis software to highlight transcriptional changes between Group A and B overlaid onto a pathway map (Appendix C). Predominant features of Group A tumours are higher transcription of proteins that can activate the PI3Kinase pathway signalling (such as extracellular matrix proteins, growth factors, tyrosine kinase receptor and insulin receptor substrate 1 adaptor protein) and TGFbeta pathway (TGFbeta). Such growth factors could also activate the MAPK pathway, but Group A tumours also demonstrate relatively lower RAS expression than Group B and over expression of MAPK signalling down regulators the MAPK phosphotases. There is also greater expression of the CREB transcription factor and NR4A1 (although this is lesssened when only squamous cancers analysed).

Features of Group B tumours appears to be higher expression of the mTOR pathway inihibitor *AMPK*. The regulation of these pathways is complex and regulated by many other means than transcription of it the extracellular regulators, but this overview suggests that cervical cancers display molecular heterogenity and that more insight into these could help to personalise therapeutic approaches.

5.10.8 The effect of HPV on the cancer cell transcriptome

The most striking feature of HPV positive tumours when compared to negative is the difference in the cell cycle and apoptosis pathway score. As expected, HPV negative tumours demonstrate lower expression of p16, as p16 expression is often used as a surrogate marker of HPV status due to the effect of the HR-HPV E7 protein in increasing p16 expression via Rb loss.

The expression of the ARF gene is also lower in HPV negative tumours, which is likely to be a reflection of loss of p53-mediated transcriptional repression of ARF in HPV tumours due to the increased p53 degradation seen in the presence of HR-HPV E6 gene.

Overall, the expression of activators of cell proliferation is greater in HPV positive tumours (MCMs, *E2F1/2/3, cycA, cdc7*). The exception to this is cyclin D, whose expression is lower in HPV positive tumours, and this is possibly a reflection of the role of p16 in transcriptional inhibition of cyclin D.

Figure 5.10 demonstrates the trend for HPV negative tumours to have lower expression of DNA repair genes than their HPV positive counterparts. A more detailed assessment of the transcriptional features of this pathway is undertaken separately, but this data is suggestive that HPV presence up-regulates DNA repair genes at a transcriptional level. Similar mRNA upregulation in HPV positive tumours were seen on a recent analysis by Prieske et al where HPV positive vulval tumours demonstrated increased transcription of HRR/BRCA/FANC pathway genes including *BRCA2, BRIP1, FANCA* and *FANCC* (206).

Whilst the mean pathway scores transcription scores for the Hedgehog pathway and the Wnt pathway do not differ significantly between the HPV positive and negative tumours, the pattern of gene overexpression in HPV negative tumours (pathway activators *Wnt, Frizzled*) and genes under expressed in HPV negative tumours (pathway inhibitors such as *PTCH1, PTCH2, DKK, FRP*) give rise to the hypothesis that the Hedgehog pathway is potentially an important driver of non-HPV related gynaecological squamous cancers, which in this cohort are predominantly vulval cancers.

5.10.9 DNA Repair Pathway gene expression in cervical and vulval cancers

Table 5.5 highlights the DNA repair pathway as one of the most dysregulated in cervical and vulval cancers. The global trend is towards an increased expression of DNA repair genes in tumour versus normal tissues, particularly those involved with homologous recombination repair. The expression of DNA repair genes is higher in HPV positive tumours (Figures 5.9 and 5.10). The differentiating genes with regards to DNA repair between HPV negative and HPV positive tumours are also in genes related to HRR, in particular the FANC pathway (Figure 5.10).

One aim of this study was to determine if there was evidence on a transcriptional level that double-strand DNA repair was inhibited in HPV positive tumours, and thus potentially accounting for their increased radiosensitivity. Figure 5.10 shows that there are some tumours demonstrating loss of expression of FANC core complex genes in relation to normal tissue (*FANCG, FANCE, FANCF, FANCC, FANCL, c19orf40/FAAP24*). This predominantly, but not exclusively, occurs in HPV negative tumours, in keeping with the lower expression of FANC proteins seen in Figure 5.10. Low expression levels of *FANCG, FANC E, FANC C* and *FANC L* predominantly occur in more advanced disease, demonstrating that perhaps downregulation of these genes could be a late event in cancer progression.

There are some interesting features in selected HPV positive tumours that may indicate that may indicate loss of FA core complex components. The most under-expressed proteins in tumours relative to normal tissues in Figure 5.10 are *c19orf40/FAAP24* in 5023T (HPV positive adenocarcinoma) and *FANCF* in 5011T (HPV positive squamous carcinoma). FANCF hypermethylation in cervical cancer samples has been previously shown (105) and therefore this may be a mechanism for low *FANCF* expression in 5011T.

Despite the increased levels of phosphorylated ATM in cervical cancers already demonstrated, there is no upregulation of ATM transcripts in cervical cancers, with a trend to reduced expression in the cancers studied. This supports the finding from previous studies in cervical cancer (126), demonstrating a significant reduction overall of ATM mRNA expression when compared to normal controls, and this has also been mirrored in this study's findings in vulval squamous cancers.

Figure 5.11 demonstrates a trend towards a negative correlation between ATM probe count and levels of cellular pATM – suggesting that high levels of pATM activation are found in cellular environments where ATM transcription is supressed. This could potentially be via negative feedback on transcriptional activity due to longevity or low turnover of pATM. Another possibility is that in untreated tissues, increased levels of pATM is a surrogate for increased double strand DNA damage as a result of a partial loss of ATM transcription due to methylation/deletion/mutation.

5.11 Discussion

This study has shown increased transcriptional activity in HPV-mediated cancers in many key genes related to DNA repair. This analysis has demonstrated that in selected cases there is a down-regulation of components of the FA core complex, but the functional consequences of this are unknown.

The lack of direct association shown between ATM expression and cellular activated (pATM) levels highlights the limitations of using transcriptional activation as a surrogate for DNA repair activity. A multitude of factors influence the proficiency of DNA repair, and regulation of this process is influenced by gene mutations, mRNA stability, post-translational modifications, subcellular protein localisation and protein degradation.

Whilst the studies performed utilising immunohistochemistry and Nanostring technology have been informative, in order to fully ascertain the functional status of double strand DNA repair in the context of HPV-related disease, functional studies are desirable. Such

studies can measure the kinetics and proficiency of DNA repair in vitro following DNA damage, which is more likely to inform regarding response to DNA-damaging treatment than in untreated tissues. The next step was to generate a novel tissue resource of primary cell lines to facilitate the study of DNA repair dynamics.

Chapter 6 – Protocol development for DNA repair assays in cervical and vulval primary cells

6.1 – Background

Double strand DNA repair is a complex and dynamic process requiring the sequential recruitment of multiple proteins to a site of damage. There has been great progress in the understanding of these processes and their impact on cancer therapies in recent years, predominantly using in vitro studies in immortalised secondary cell lines (207). Secondary cell lines have the advantage that they are generally robust on multiple passaging and grow in a predictable fashion, making them easier to manipulate and facilitate in-vitro assays. There are multiple common commercially available cervical and vulval cancer cell lines (Table 6.1), but due to extensive use over many years in the in vitro environment it is likely that cellular phenotypes are no longer be representative of the tissue from which they were derived due to the selective pressures and acquired mutations during multiple passaging. Genetic drift within a cell line can lead to rapid genetic diversification, with different strains from the same cell line giving differential responses to in vitro treatments (208). Studies of commonly used ovarian cancer secondary cell lines have demonstrated significant changes in copy-number, mutations and pronounced differences in mRNA expression profiles between the cell lines and ovarian cancer tumour samples (209).

It was therefore a priority to produce better in vitro models to evaluate the kinetics of DNA DSBs in the context of gynaecological HPV-mediated cancers. Primary cells explanted from fresh tumour tissue at early passages are potentially more likely to demonstrate similar biological characteristics as the tissue of origin, including DNA repair. The aim of the work contained in this chapter was to develop primary cell resources from cervical and vulval tissue, characterise them and determine their utility in assays of DNA DSB repair proficiency. A comparative analysis of the RNA transcriptome of the primary cells and donor tumour was also planned to determine how the in vitro environment altered transcriptional signatures of common cancer-associated pathways in the PanCancer codeset. Whilst many of these aims were accomplished, I was unable complete a planned series of experiments to analyse the functional status of DNA repair in HPV positive and HPV negative primary cells due to COVID-19 pandemic disruptions. This work has been successful in providing a model for future studies.

Table 6.1 Common commercially available cell lines for the study of cervical and vulval cancer

	Tumour of origin	Site of origin	HPV Status	Year Established
Hela	Cervical adenocarcinoma	Cervix	HPV 18	1951
Caski	Cervical squamous cancer	Small Intestine	HPV 16	1977
Siha	Cervical squamous cancer	Cervix	HPV 16	1970
C33a	Cervical squamous cancer	Cervix	Negative	1964
A431	Vulval squamous cancer	Vulva	Negative	1973

6.2 Recruitment and Tissue for Primary Cell Line Development

Fresh tissue samples were obtained from individuals undergoing planned procedures under general aneasthesia for management of histologically confirmed vulval and cervical cancers at Liverpool Women's Hospital and Clatterbridge Cancer Centre (REC 15/NW/0425) (Appendix A). An overview of the study protocol is outlined in Figure 6.1 and the clinicopathological data of the study participants recruited for fresh tissue collection is shown in Table 6.2.

6.3 Optimisation of cell culture protocols

Previous work performed within the group had developed a protocol (Protocol 1, Figure 6.2) to develop squamous cell cancer primary cells from HPV positive oropharyngeal cancers utilising mechanical dissociation of primary tissue followed by adherence to CellBind wells and incubation in selective media (135). However, this protocol had failed to generate cultures expressing HPV proteins from HPV positive oropharyngeal primary tumours, and therefore a second protocol was also used (Protocol 2, Figure 6.2), developed by a collaborator, Dr N Powell (Cardiff), who had successfully generated HPV expressing cultures from vulval and vaginal dysplastic lesions utilising a feeder layer of 3T3 fibroblasts (210) . Protocol 2 utilised GMEM media (with epidermal growth factor (EGF)

supplementation from day 4). Both of these approaches were utilised in parallel within the study (full protocols are found in Appendix A).

Each study participant was given a unique study number 50XX and the cell lines were named after this study number with the annotation ‘T’ for tumour tissue culture ‘N’ for adjacent normal tissue culture. When sizeable explants formed, they were removed by trypsinisation and transferred to a tissue culture flask labelled passage 1. The well from which they originated on the CellBind plate was added to the primary cell name. In the case of culture dishes with feeder cells the fibroblasts were removed mechanically by pipetting with PBS or via differential trypsinisation.

Table 6.2 Clinicopathological data of study participants for primary cell development

Study number	Tissue origin	Age	Stage	Primary Treatment	Treatment response	Disease Recurrence
5001	Squamous cancer cervix	29	2B	Surgery + pelvic EBRT with brachytherapy	CR	No
5002	Squamous cancer cervix	77	1B1	Surgery	CR	No
5003	Squamous cancer vulva	75	3A1	Surgery + EBRT	CR	No
5004	Squamous cancer cervix	71	2B1	Chemoradiation	CR	No
5005	Squamous cancer cervix	29	1	Surgery	CR	No
5006	Squamous cancer vulva	71	1	Surgery	CR	No
5007	Squamous cancer vulva	74	3	Surgery + EBRT	PD	Yes
5008	Squamous cancer vulva	80	recurrent	Surgery	PD	Yes
5009	Squamous cancer vulva	73	2	Surgery	CR	No
5010	Squamous cancer cervix	53	3B	Chemoradiation	PR	Yes
5011	Squamous cancer cervix	42	2B1	Chemoradiation + brachytherapy	PR	Yes
5012	Squamous cancer cervix	36	1	Surgery	CR	No
5013	Squamous cancer cervix	62	1B1	Surgery + EBRT	CR	No
5014	Squamous cancer cervix	70	1B1	Surgery	CR	No

CR – complete response, PR – partial response

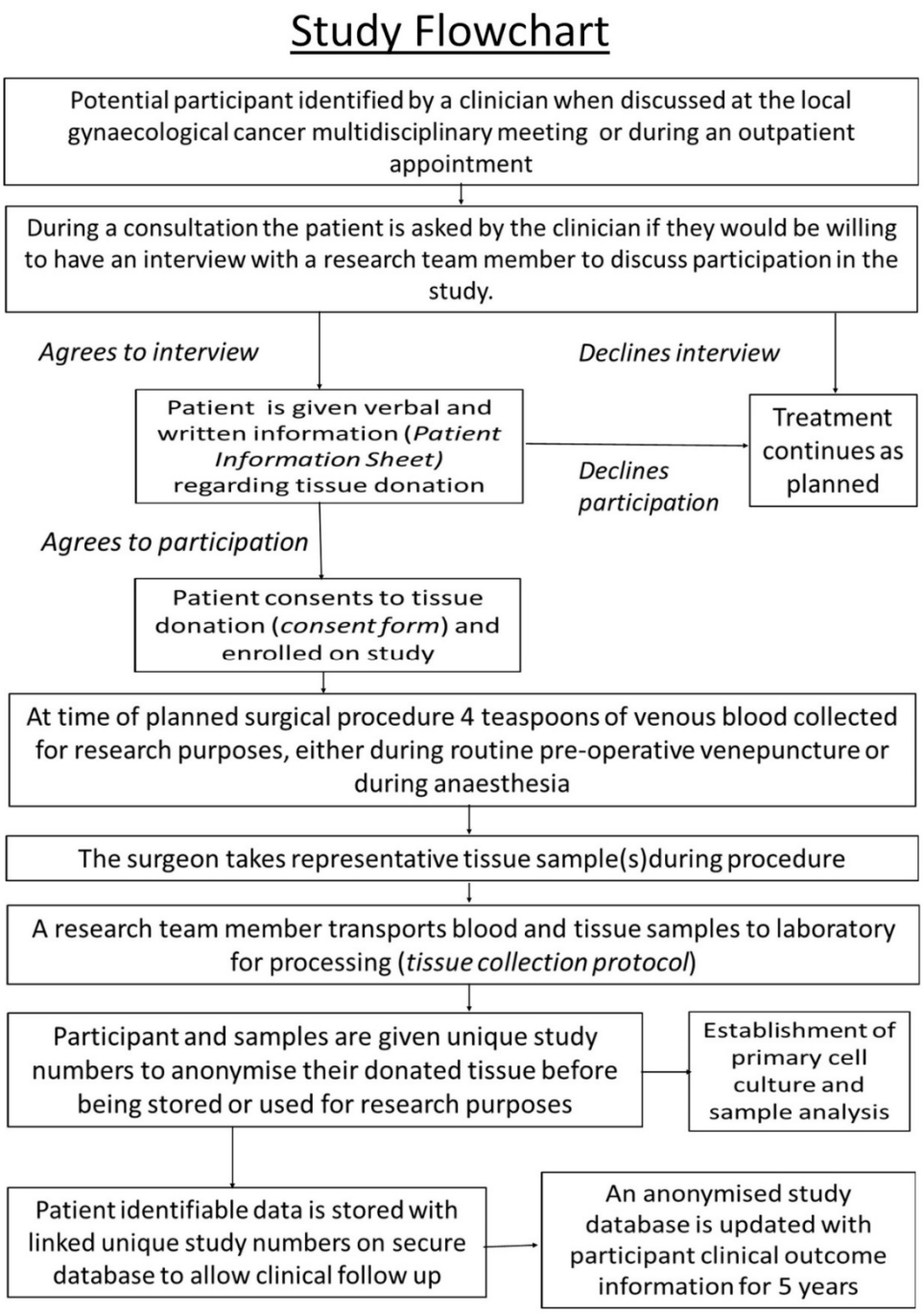


Figure 6.1 Study Protocol - Tumour tissue procurement to allow establishment of primary cell cultures for use in Human papillomavirus-mediated cancer research

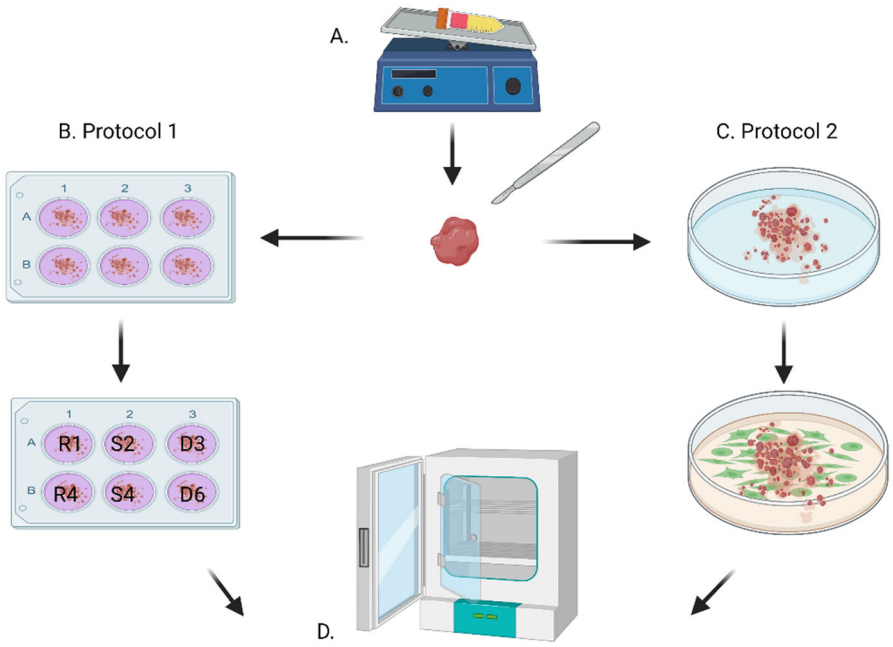


Figure 6.2 Protocols to generate Primary Cells

A. Tissue washed in transport medium (DMEM with 10% fetal bovine serum (FBS), 1% L-Glutamine and 2% penicillin/streptomycin) for 60 minutes (with media change every 20 minutes) B. Explanted tissue dissected into small pieces (1-2mm^3) in a few drops of transport media, divided between six wells of a CellBind plate and incubated for 1hour at 37^{o}C with 5% CO_2. Media then added dropwise to each well as annotated (R=RPMI, S=SFKM, D=DMEM) until tissue covered. Media topped up to 3mls after 36-48hours. C. Explanted tissue rinsed with FBS, and tissue dissected into small pieces (1-2mm^3) in a few drops of FBS and dish inverted for 1hour at 37^{o}C with 5% CO_2. 3mls of GMEM media then added (without EGF) with 5 x 10^5 irradiated 3T3 cells. D. Cells incubated at 37^{o}C with 5% CO_2 for 4 days.

6.3.1 Initial observations on cell development protocols

The first three tissue sample collections (5001-5003) were processed for primary cell culture as shown in Figure 6.2. These cases demonstrated some areas where protocol modifications were to be made to improve the process of primary cell production.

a. Fungal contamination - several wells and flasks were discarded due to this issue, which seems particularly prevalent when small tissue culture dishes were used in protocol 2, perhaps due to difficulties in handling these without disturbing the lid. It is likely that some of the contamination was due to colonisation of the primary tissue samples, which were washed with antibiotics (penicillin and streptomycin in the transport media) but were not exposed to anti-fungal agents such as Amphotericin as previously published data suggests that Amphotericin exposure may influence HPV expression (210). To overcome some of these issues, NUNC coated 6 well plates (Fisher scientific) were adopted instead of small TC dishes.
b. Cell senescence - following initial adherence and growth of keratinocyte populations in CellBind wells with SFKM the cells would become senescent following prolonged incubations. In such circumstances it was decided to trial the addition of a feeder layer to the well to stimulate keratinocyte explant growth.
c. Mixed cell type populations – once keratinocytes and fibroblasts coexisted in RPMI media and DMEM:F12 media in cell bind plates they were very difficult to effectively separate. Differential trypsinisation and cell scraping were utilised to separate fibroblasts from keratinocytes, but the fibroblasts were difficult to completely clear and could quickly overgrow keratinocyte populations. As the interest of this project lay in the primary keratinocytes populations it was decided to only use SFKM and GMEM media (with EGF supplementation after 4 days) to encourage the preferential growth of keratinocytes.
d. The irradiated 3T3 feeder cells used for protocol 2 would detach after short periods of time and need replenishing regularly. A robust, primary, human fibroblast cell line was developed from the 5001 explants, and this was trialled to see if these early passage cells may provide a better feeder layer once irradiated, with the additional benefit of being more representative of cervical stromal tissue.

6.3.2 Protocol optimisation

Two new protocols were devised for tissue collections from 5004 onwards to incorporate these changes (Protocol 3 and 4, Figure 6.3). If there was poor growth of keratinocytes in a CellBind well with SFKM, 5001T irradiated fibroblasts were added as a feeder layer.

The trial of irradiated 5001T primary fibroblasts as a feeder layer was successful, and it was superior to the irradiated 3T3s cells in maintaining adherence, only needing to be replenished after several weeks of culture. When a series of fresh tissue samples were grown on 5001T irradiated fibroblasts in SFKM, keratinocyte explants were seen (Figure 6.4). When the GMEM media was utilised, both primary keratinocyte and fibroblasts were seen (Figure 6.4). As the process of separation of explanted fibroblasts/irradiated fibroblast feeder cells from tumour explants was time consuming the preference, if possible, was to grow explant keratinocyte cultures without feeder cells in SFKM if the cells continued to proliferate and maintained HPV positivity during multiple passage. The 5005T sample collection demonstrated that both normal and tumour keratinocyte cultures could be grown in these circumstances (Figure 6.5). Protocol 3 became the protocol of choice for generating cell lines for subsequent tissue collections.

6.4 Non-Adherent Primary Culture

All tissue samples collected led to the generation of adherent cell cultures except tissue from patient 5011, which formed a mixed population of adherent fibroblastic cells and cells dividing in suspension as demonstrated in Figure 6.6. The cells in suspension loosely attached to the adherent fibroblasts where mixed populations existed but also continued to grow in suspension, even if an irradiated 5001T feeder layer was introduced. As such these cells would be difficult to analyse in assays such as immunofluorescence, and an attempt to culture them as spheroids was undertaken to provide a model that could undergo formalin fixation and paraffin embedding. Unfortunately attempts at growing 5011T spheroids in ultra-low attachment (ULA) wells were not successful. 5005T early passage cells formed small spheroids (Figure 6.6) but this mode of culture was not pursued further due to difficulties keeping the small spheroids intact during fixation and embedding.

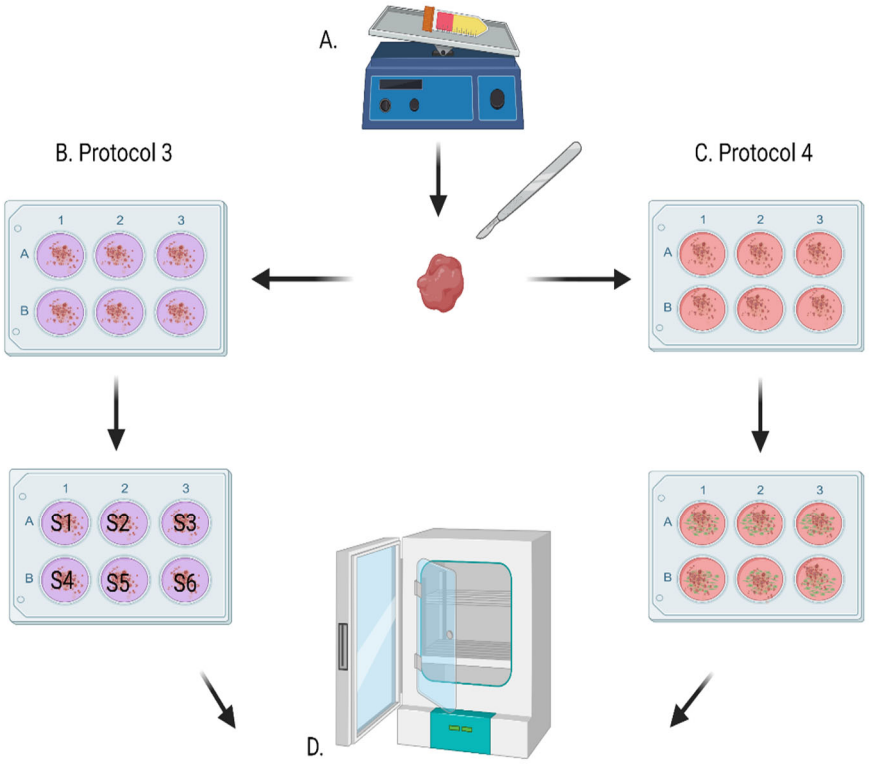


Figure 6.3 Optimised Protocols to generate Primary Cell Lines

A. Tissue washed in transport medium for 60 minutes (with media change every 20 minutes) B. Explanted tissue dissected into small pieces (1-2mm) in a few drops of transport media, divided between six wells of CellBind plate and incubated for 1hour at 37°C with 5% CO_2. SFKM was then added dropwise to each well until tissue covered. Media topped up to 3mls after 36-48hours. C. Explanted tissue rinsed with FBS, tissue dissected into small pieces (1-2mm^3) in a few drops of FBS and placed in six wells of NUNC coated plate for 1hour at 37°C with 5% CO_2. 3mls of GMEM media (without EGF) was then added with 5 x 10^5 of irradiated 5001T fibroblast cells. D. Cells incubated at 37°C with 5% CO_2 for 4 days.

Table 6.3 Summary of Primary Cell Line development

Study number	Tumour Type	Tissue HPV status	Cells developed	Highest passage number to date
5001	Squamous cervix	uk	Tumour fibroblasts	P12
5002	Squamous cervix	+	n/a	n/a
5003	Squamous vulval	-	n/a	n/a
5004	Squamous cervix	+	Tumour keratinocytes	P4 (senescent)
5005	Squamous cervix	+	Tumour keratinocytes	P15
		-	Normal keratinocytes	P6
5006	Squamous vulval	uk	n/a	n/a
5007	Squamous vulval	-	Normal keratinocytes	P3
5008	Squamous vulval	-	Tumour fibroblasts	P3
5009	Squamous vulval	-	Tumour keratinocytes	P9
5010	Squamous cervix	uk	n/a	n/a
5011	Squamous cervix	+	Tumour keratinocytes	P2
5012	Squamous cervix	uk	n/a	n/a
5013	Squamous cervix	-	Tumour keratinocytes	P3 (senescent)
5014	Squamous cervix	uk	Tumour keratinocytes	P5

uk=unknown (insufficient tissue/RNA yield for reliable PCR) P = passage

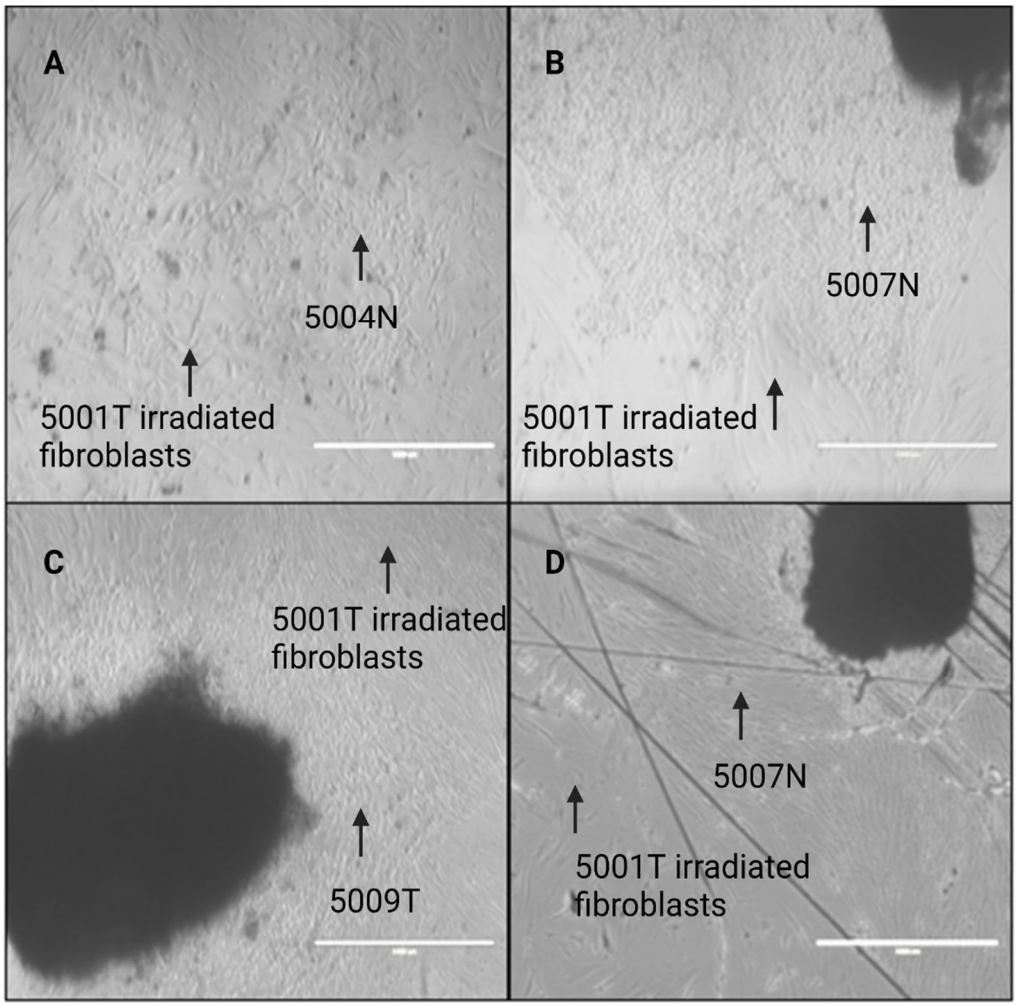


Figure 6.4 Examples of cell growth on 5001T irradiated feeder cells

A.5004N keratinocyte colony passage1 grown with 5001T irradiated fibroblasts (SFKM).

B. 5007N keratinocyte explant on 5001T irradiated fibroblasts grown (SFKM).

C. 5009T keratinocyte explant on 5001T irradiated fibroblasts grown (GMEM).

D.5007N primary fibroblast explant on 5001T irradiated fibroblasts grown (GMEM).

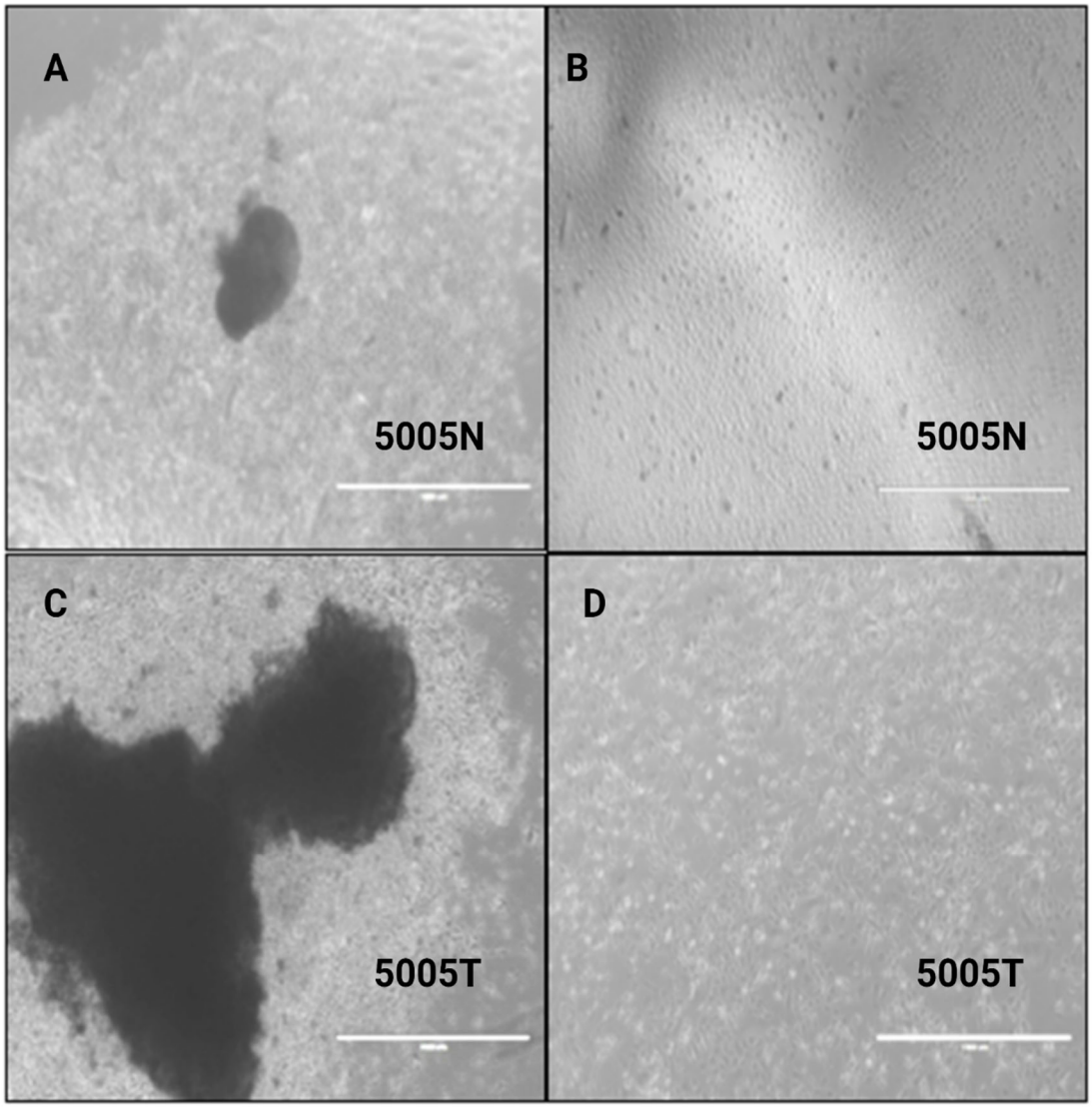


Figure 6.5 Examples of cell growth in the absence of a feeder layer

A. 5005N keratinocyte explant on day 7 (SFKM)

B. 5005N primary keratinocytes passage 1 (SFKM).

C. 5005T primary keratinocyte explant on day 7 (SFKM).

D. 5005T primary keratinocytes passage 1 (SFKM).

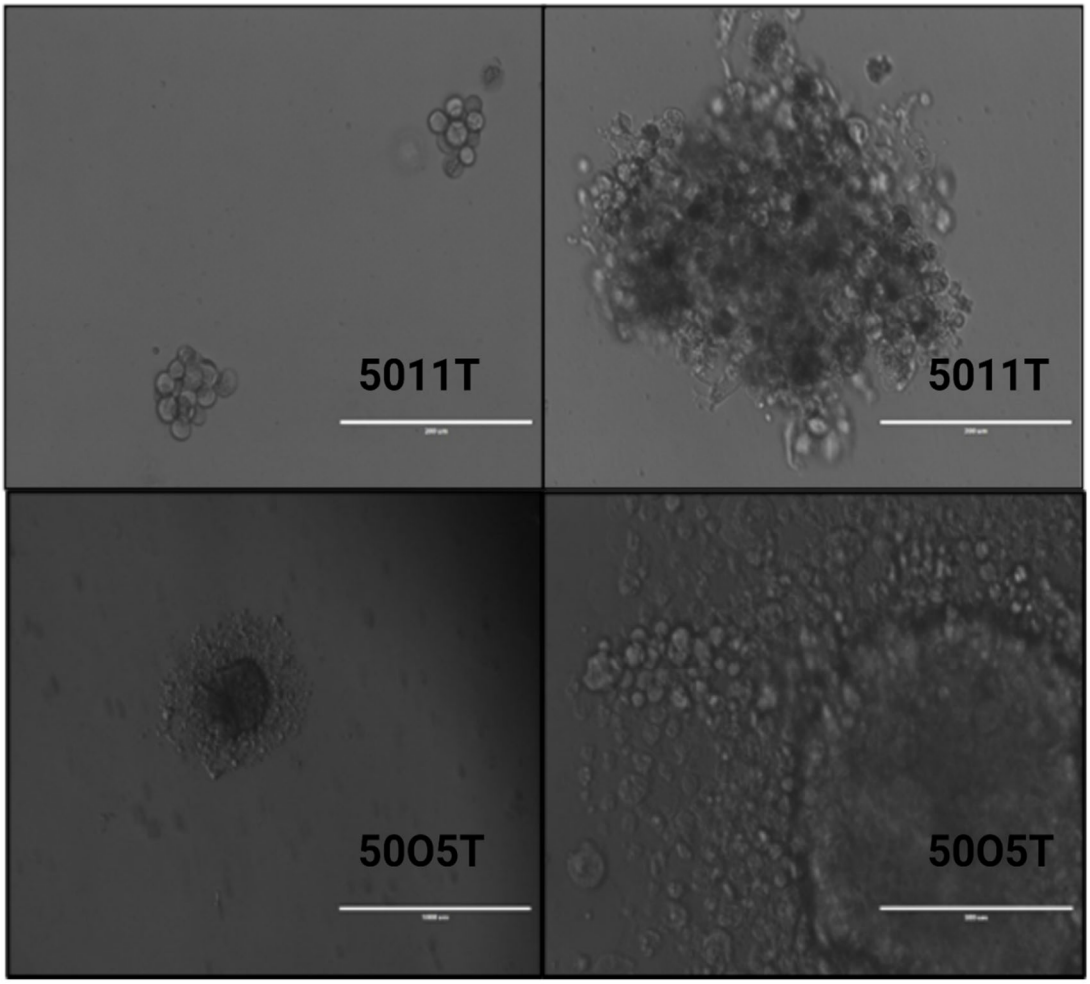


Figure 6.6 Primary cells growing in suspension

A. 5011T passage 1 (smaller colonies)

B. 5011T passage 1 once colony reached maximum size.

C. and D. 5005T Day 3 spheroid culture.

6.5 Cell Characterisation

6.5.1 Cell morphology and pancytokeratin expression

After completion of 14 tissue collections, there were 3 cell lines which emerged which withstood multiple passaging and were selected as the most robust for use in-vitro assays: 5005N, 5005T, 5009T (Table 6.3). They were morphologically in keeping with keratinocytes (Figure 6.4, Figure 6.5), and pancytokeratin expression was confirmed by immunofluorescence (Figure 6.7).

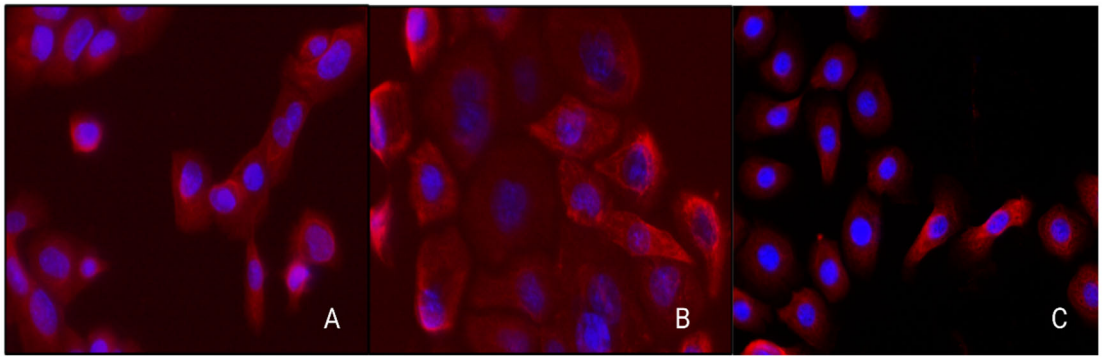


Figure 6.7 Primary Cell Immunofluorescence staining with Pancytokeratin antibody

A. 5005T

B. 5009T

C. Normal oral keratinocyte (NOK) control

6.5.2 Mycoplasma Testing

All cell Lines underwent PCR for mycoplasma testing and were negative (performed by Institute of Translational Medicine, Mycoplasma Screening Service).

6.5.3 STR profiling

To confirm that the cells grown through multiple passages originated from the source tumour sample, Short Tandem Repeat profiling was undertaken on DNA extracted from the original tissue sample (fresh frozen at time of collection) and the primary cells during passage. The STR profiling was performed by The Institute of Translational Medicine STR profiling service with the Promega GenePrint 10 system. All primary cells demonstrated identical STR profiles to the tissue of origin (Table 6.4).

Table 6.4 Short Tandem Repeat Data on Primary Cells and Corresponding Tissue Sample

	5005N		5005T S5P4		5005N S2P3		5009T		5009T S2P11	
THO1	7	7	7	7	7	7	8	9.3	9.3	9.3
D21S11	31	32	31	32	31	32	30	32.2	30.2	30.2
D5S818	11	12	11	12	11	12	11	13	11	13
D13S317	11	12	11	12		12	11	11	11	11
D7S820	11	12	11	12	11	12	10	10	10	10
D16S539	11	12	11	12	11	12	11	12	11	12
CSF1PO	10	13	10	13	10	13	10	10	10	10
AMEL	X		X		X		X		X	
vWA	17	18	17	18	17	18	14	19	19	19
TPOX	8	9	8	9	8	9	8	9	8	9

6.5.4 – HPV Testing

RT qPCR was used to test for HPV 16/18 positivity. This analysis demonstrated HPV 18 positivity of the 5005T donor tissue with similar levels of expression to the Hela control ($2^{-\Delta\Delta CT}$ 0.6). Analysis of subsequently passaged cell lines (5005TS5 passages 6, 9 and 13) demonstrated ongoing HPV 18 expression, with a comparable expression of HPV 18 E6. Tissues 5005N, 5009T and 5009T primary cell did not express HPV 18 E6 or HPV 16. To confirm whether the 5009T tissue or cells exhibited infection with less common subtype of HR-HPV (31, 33, 45), formalin fixed tumour and primary cell pellets were analysed by HPV RNA ISH (M. Robinson, Newcastle). This demonstrated the absence of HR-HPV transcripts in 5009T tumour tissue, 5009TS2 and 5009TS5 primary cells.

6.5.5 Transcriptomic profiling of primary cells compared to tissue of origin

RNA extracted from primary cell cultures 5005T, 5005N and 5009T was analysed on the Nanostring platform using the PanCancer codeset as previously described in Chapter 5. A summary of pathway scores for the samples is demonstrated in Figure 6.8. This demonstrates clustering of signatures between primary cells and their tissue of origin, suggesting that characteristics are retained. The adjacent normal cells from 5005 tissue collection demonstrate separation from tumour tissue and tumour derived primary cells. There is a pattern of high DNA repair protein expression in HPV positive tumour tissue

(5005T) and primary cells originating from this in contrast with 5005 cells derived from normal tissue and from HPV negative vulval tumour cells derived from 5009T tissue.

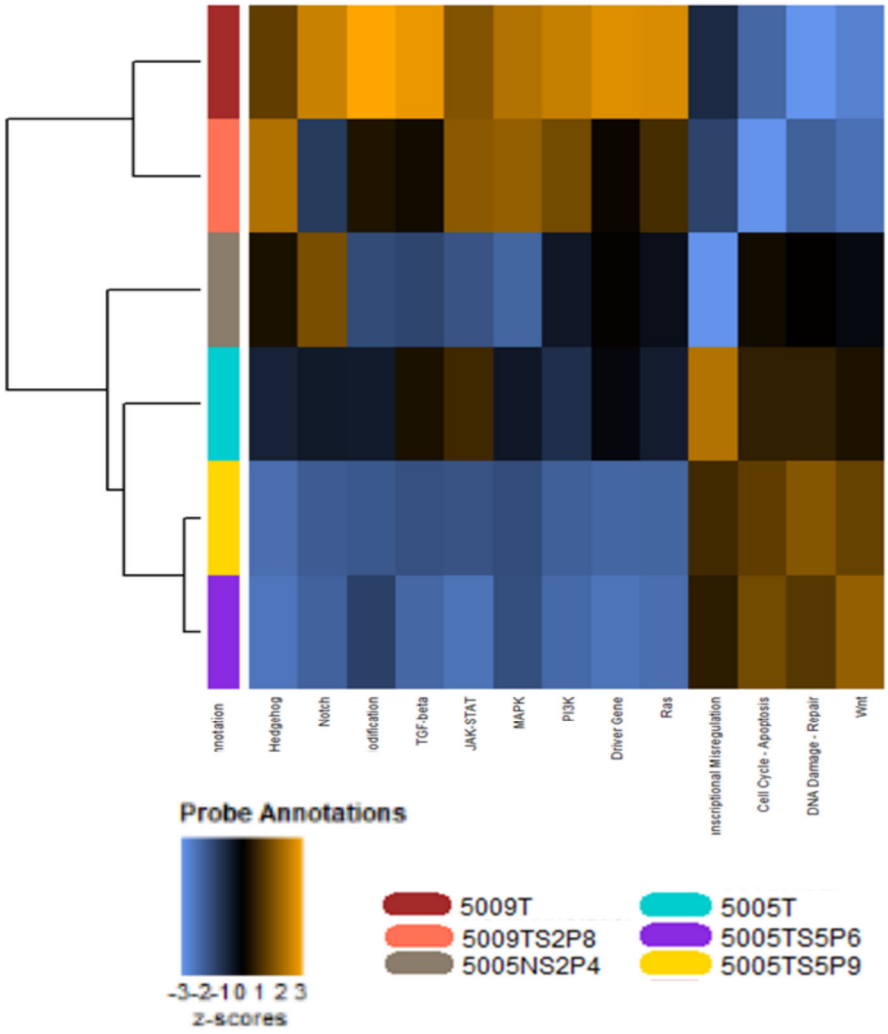
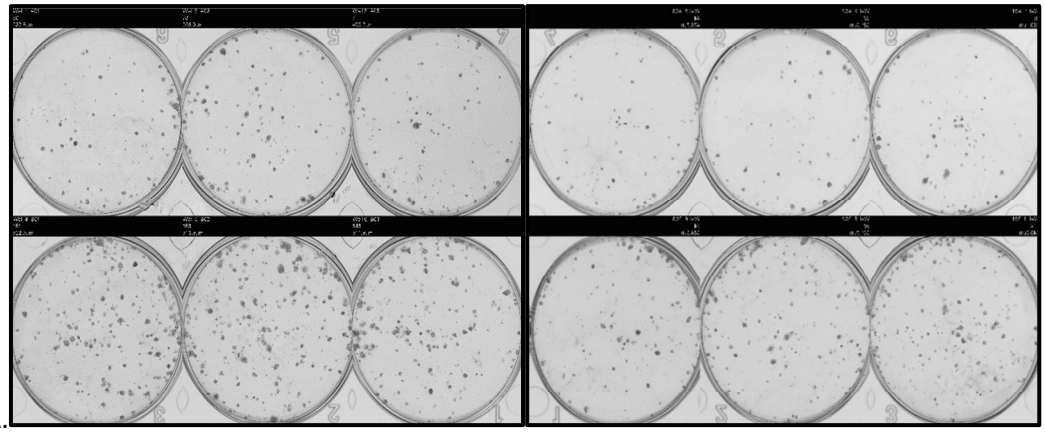


Figure 6.8 Heatmap of cancer pathway scores by cell type

6.6 – Functional studies of DNA DSB repair in primary cells

6.6.1 Clonogenic survival studies

Clonogenic survival studies were performed on the primary cells from the 5005T explanted tissue and compared to a secondary cervical cell line (siha). These studies reflect the ability of cells to withstand ionising radiation and retain the potential to replicate (as a surrogate marker of treatment response to radiation treatment). Example images and the cell survival curves from triplicate experiments are shown in Figures 6.9 and 6.10. The results indicate that 5005T is potentially more radiosensitive than siha, although the confidence intervals are overlapping. There were significant challenges when performing these assays with primary cells due to very low plating efficiencies seen with the primary cells. The average plating efficiency (proportion of untreated cells that grew into a colony) for 5005T was 0.05, compared to 0.39 for siha, requiring very large numbers of cells to be available for each assay in the primary cell population. This was not possible with the other primary cells developed and attempts at plating with lower numbers resulted in very low plating efficiencies, highlighting the lower capacity of primary cells to develop colonies from single cells populations over a 2 week period of incubation when compared to transformed secondary cell lines.

A.  B.

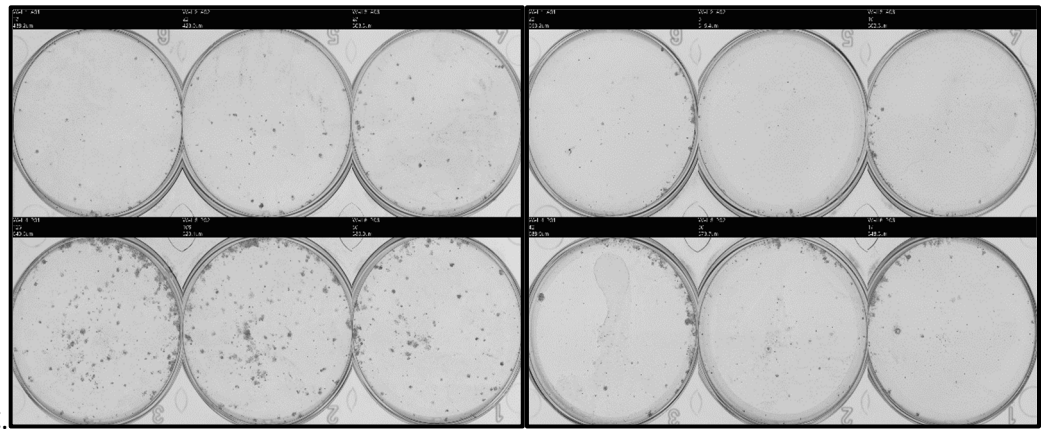
C.  D.

Figure 6.9 Clonogenic Assay plates for 5005T primary cells

A. 0Gy irradiation 1000 cells plated/well (top row) and 2000 cells plated/well (bottom row)

B. 1Gy irradiation 1500 cells plated/well (top row) and 3000 cells plated/well (bottom row)

C. 2Gy irradiation 2000 cells plated/well (top row) and 4000 cells plated/well (bottom row)

D. 4Gy irradiation 4000 cells plated/well (top row) and 8000 cells plated/well (bottom row)

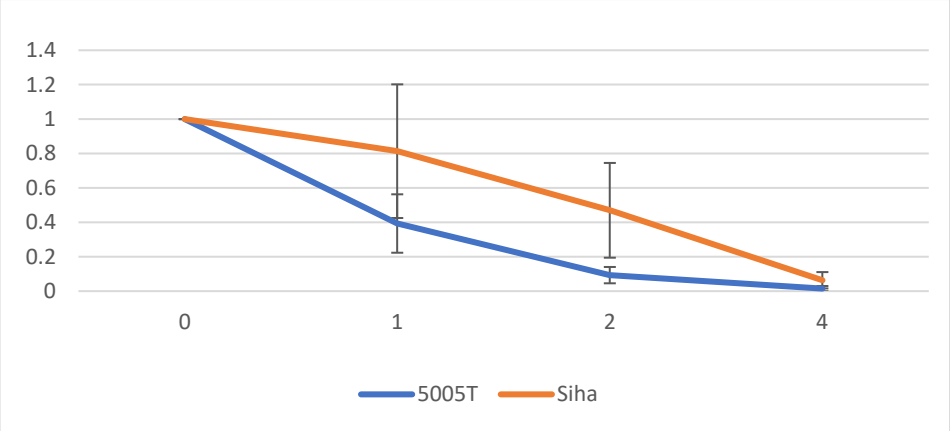


Figure 6.10 Clonogenic Assay survival curve. X axis amount of radiation (Gy). Y axis demonstrates relative colony formation (surviving fraction) expressed as colonies per treatment level versus colonies that appeared in the untreated control.

6.6.2 Immunofluorescence studies

A different type of assay to determine radiosensitivity was therefore desirable which could take place over a shorter time frame and was less reliant on the sustained proliferative capacity of the primary cells in vitro. Immunofluorescence studies of primary cells at fixed timepoints post irradiation were planned with two aims. Firstly, to evaluate the relative expression of RAD51 foci in cells treated with DNA damaging therapy versus untreated cells, which has previously been described as a surrogate marker of HRR proficiency in the primary cells (211). Secondly to assess the kinetics of pATM foci formation and resolution following ionising radiation in vitro, as data in Chapter 4 is suggestive of potentially lower expression of pATM activity in some tumours compared to normal tissues.

A protocol was adopted from M Price (Manchester) to assess RAD51 foci at baseline and 2 hours following ionising radiation. A fold change of 2 or above for average number of RAD51 foci/cell 2 hours post irradiation versus untreated cells was adopted as a marker of HRR proficiency. This assay was performed on 5005T cell lines, with at least 100 cells analysed per chamber and an average taken of the duplicate chambers per experiment (Figure 6.11). γH2AX co-staining was performed in one chamber/slide as a marker of DNA DSB. Average fold change after 4Gy post irradiation was over 2, indicating proficiency of HRR response to DNS DSBs (Table 6.5). A run performed with 2Gy irradiation versus 4Gy appeared to demonstrate similar levels of DNA DSBs as indicated by γH2AX foci, but a

lower fold change of RAD51. This was likely due to the high baseline levels of RAD51 counted in untreated cells in this run, which was approximately 2 to 3-fold that seen in the untreated cells in the 4Gy experiments. The reason for this is not clear and further work would be desirable in this and other cell lines to determine homologous recombination repair status.

Table 6.5 Immunofluorescence data for RAD51/ γH2AX staining in untreated cells and those 2 hours post irradiation with 2Gy and 4Gy

	RAD51			**γH2AX**		
	Untreated	**2 hours post irradiation**		**Untreated**	**2 hours post after irradiation**	
	Average foci/nucleus	Average foci/nucleus	Fold change	Foci/nucleus	Foci/nucleus	Fold change
4Gy						
Run 1	1.63	3.77	2.31	1.64	5.98	3.64
Run 2	1.03	2.92	2.83	0.66	2.19	3.31
2Gy						
Run 1	3.02	2.45	0.81	1.21	5.12	4.23

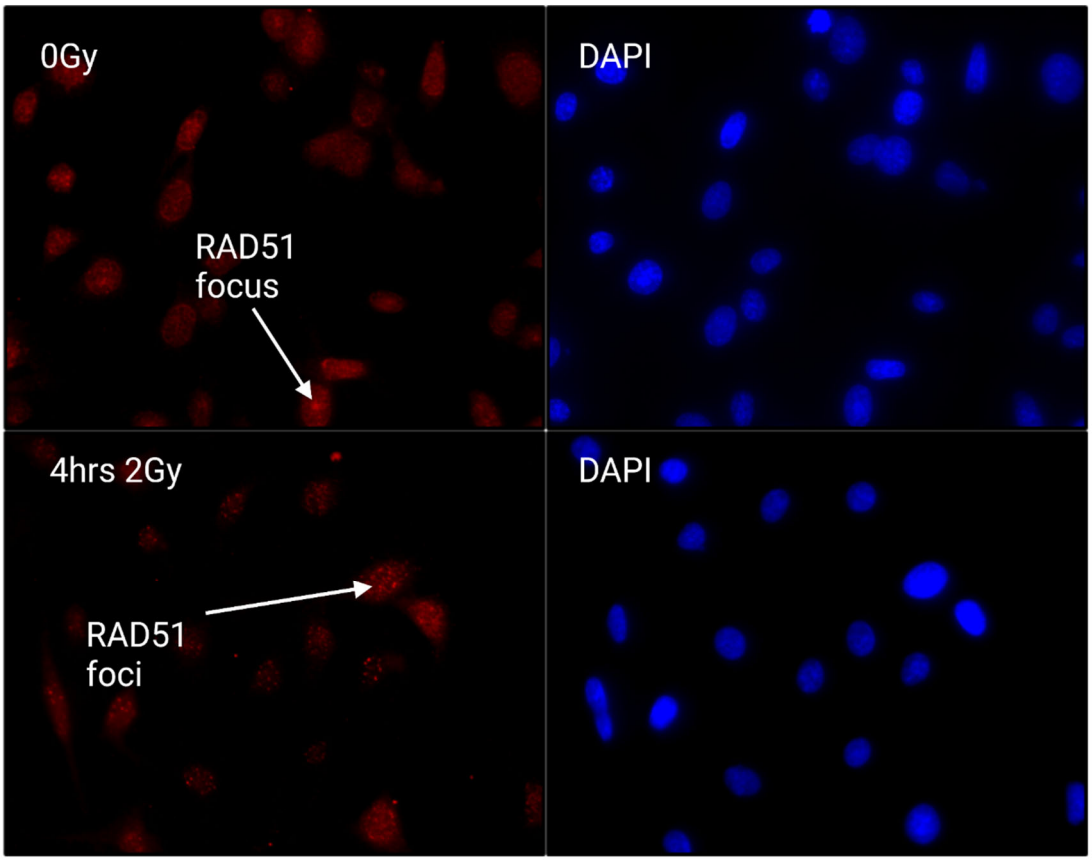


Figure 6.11 RAD51 cell immunofluorescence in untreated 5005T cells (0Gy) and 5005T cells 2 hours post 4Gy irradiation with corresponding DAPI stain showing cell nuclei

Immunofluorescence for pATM was also performed with 5005T cells to determine if pATM foci were appropriately formed on the induction of DNA damage. Previous work in the study had characterised 5005T tumour as expressing high levels of nuclear pATM (score 5) and low levels of cytoplasmic pATM (score 3) while ATM RNA probe counts in 5005T baseline tumour are lower than normal cervical controls (Figure 5.10).

An assessment of pATM foci formation was made at baseline and then at 1, 2, 4, and 8hrs after 2Gy-4Gy ionizing radiation to determine if pATM foci were appropriately induced post treatment, and if there was any evidence of prolonged pATM foci persistence following treatment.

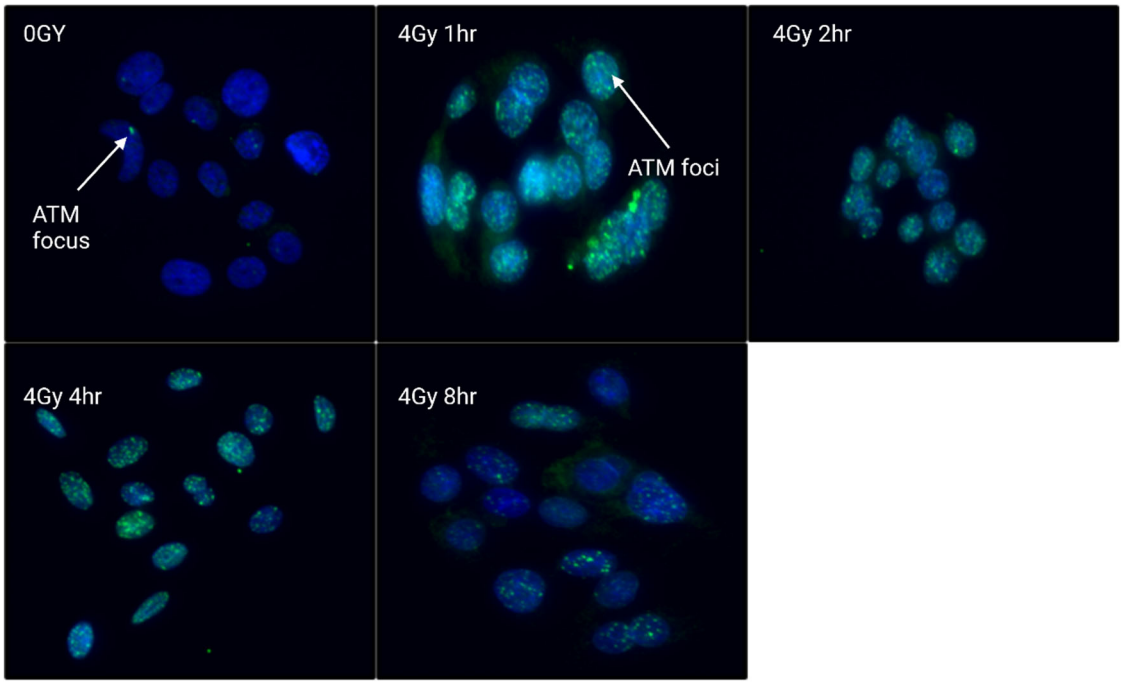


Figure 6.12 Images of pATM immunofluorescence in untreated cells (0Gy) and at 1, 2, 4 and 8hrs following 4Gy ionising radiation

These data demonstrate early foci formation of pATM and resolution of pATM foci in line with γH2AX resolution (Figure 6. 13). This is indicative of proficient ATM activity in the 5005T cell line, reflective of the high pATM activation seem on immunohistochemistry. It supports earlier findings of a poor correlation between ATM RNA levels and levels of nuclear ATM activation by phosphorylation.

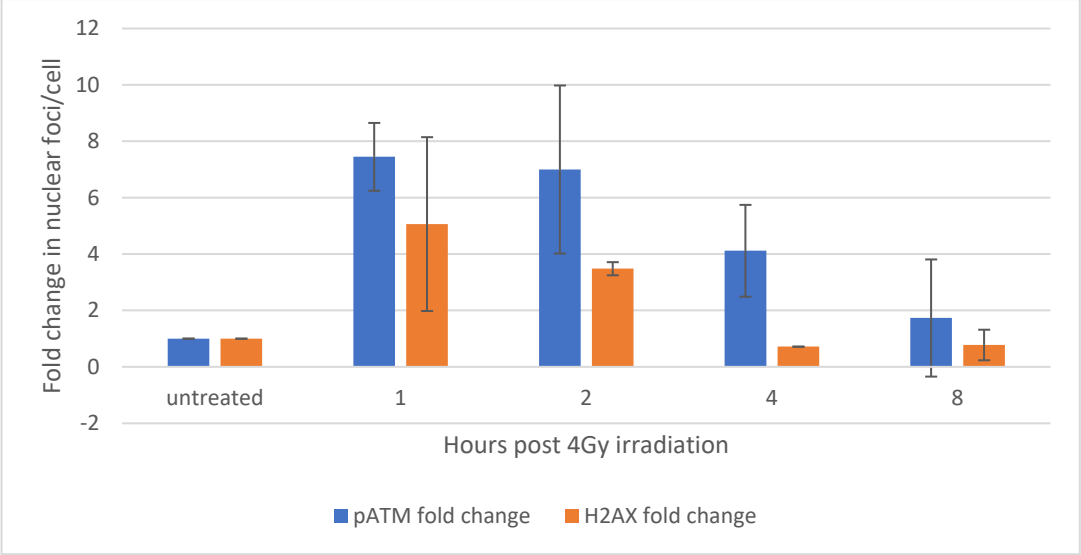


Figure 6.13 Average fold change in pATM nuclear foci/cells in untreated cells and following 4Gy at 1, 2, 4, 8 hours

6.6.3 Immunofluorescence on FFPE tumour tissue

Whilst the development of primary cells is good model for exploring homologous recombination proficiency in vitro, the logistical constraints, time required and potential failure of explants to yield proliferating cells makes it challenging to use primary cell functional assays as a predictive therapeutic marker to predict response to radiation in clinical practice. Several studies have demonstrated the predictive therapeutic value of assessment of RAD51 foci on pre-treatment and post treatment (24hr-36hr) biopsies in breast and head and neck cancers treated with DNA damaging chemotherapy (133, 212). In these studies, biopsies retaining RAD51 foci levels >10% from baseline at 24-36 hours post-treatment were assigned to have delayed DNA DSB kinetics and were associated with improved treatment outcomes.

More recent studies have investigated if an assessment of RAD51 foci in untreated diagnostic biopsies alone can be a surrogate marker of HRR deficiency and/or predictive for treatment response to DNA damaging agents. Such an approach has clear advantages in terms of ease of clinical use but lacks the use of exogenous DNA damage delivered in previous studies that perform RAD51 assessment. A study in ovarian cancer has demonstrated that tumour with lower baseline RAD51 foci in replicating cells (staining positive for geminin) in FFPE tissue may be indicative of HRR deficiency and improved

response DNA damaging therapy (213). It is possible that the higher levels of DNA damage seen in the presence of HR-HPV infection may provide sufficient genomic instability to make a discrimination between cells that appropriately form RAD51 foci and those who do not. Figure 5.10 demonstrates the globally increased expression of RAD51 in cervical and vulval tumours compared to normal tissues, but to form RAD51 foci at sites of DNA repair requires upstream HRR pathway functionality and can act as a marker for the proficiency of the pathway. As a pilot study, FFPE sections from cervical cancers were co-stained for RAD51 and geminin immunofluorescence and the percentage of geminin positive tumour cells which displayed 1 or more RAD51 foci were evaluated in a minimum of 350 cells. Example images are seen in Figure 6.14.

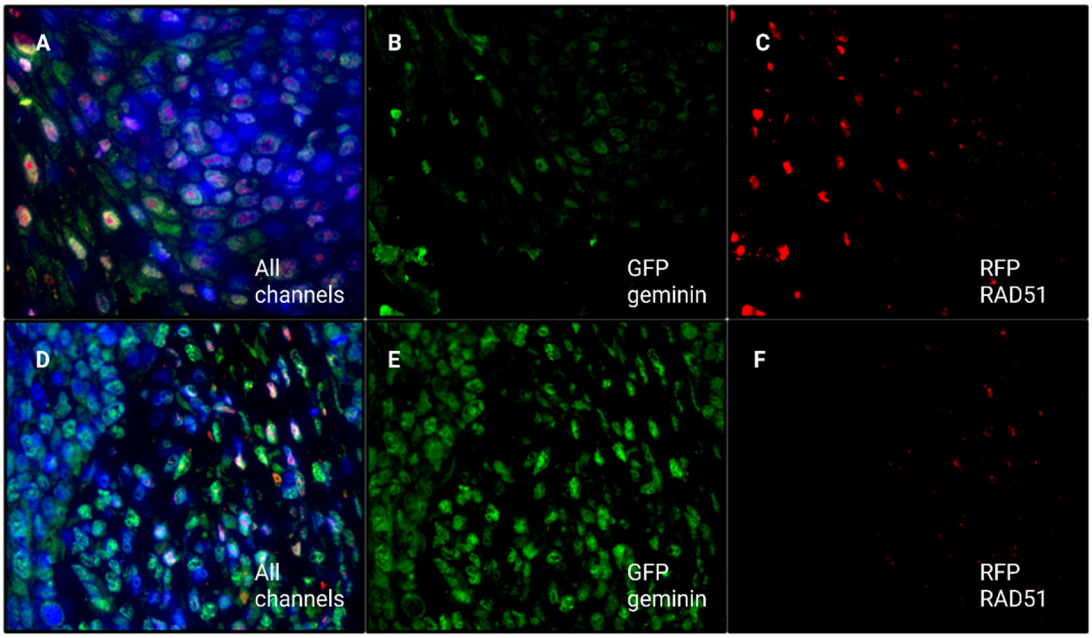


Figure 6.14 Immunofluorescence staining on cervical tumour tissue

Case 1 (A-C). 60% of geminin positive cells demonstrated RAD51 foci

Case 2 (D-F). 30% of geminin positive cells demonstrated RAD51 foci

6.7 Discussion

This study has refined a technique for growing primary cells from explanted cervical and vulval tissue and has demonstrated that these cells retain expression of HR-HPV oncoproteins during in vitro culture and display transcriptomic profiles similar to the parent tissue with regards to key cancer-associated pathways. They therefore represent a valuable and representative model of tumour behaviour under the right conditions. Challenges were experienced in the use of primary cells in *in vitro* assays, as many that grew initially did not retain proliferative capacity over multiple passages which resulted in difficulties when performing functional assays such as clonogenic survival studies. This problem was exacerbated by performing much of the tissue collection in advance of the planned experiments and, when frozen cells were resurrected after a period in liquid nitrogen, the fraction of proliferative cells was further reduced in some cases. This highlights the advantages of prospective collection for immediate use of early passage cells in assays whilst they retain good levels of replication.

Limited studies of the HPV 18 positive 5005T cell line demonstrates probable HRR proficiency based on RAD51 assay after 4Gy radiation. Further studies would be desirable to confirm this due to inter-experimental variability. To further assess the utility of this assay as a predictive tool for response to DNA damaging therapies, a prospective collection of explanted tissue for immediate evaluation of early passage primary cells by immunofluorescence was planned, focussing on locally advanced tumours undergoing chemoradiation so that corresponding response data was available. Such a series of experiments was prohibited by the COVID-19 pandemic occurring at the time assigned for further cell assays. Comparative studies between HPV positive and HPV negative vulval tumours would also be desirable to further knowledge about a hypothesised differential response in DNA repair between the two subgroups.

Work in this thesis has demonstrated that low nuclear pATM confers a survival advantage in OPSCC treated with DNA damaging therapies as part of their treatment plan, and that a proportion of cervical cancers exhibit lower pATM via immunohistochemistry than adjacent normal tissue. It has also shown that ATM is not upregulated in cervical cancers, unlike many other HRR genes and, as a key sensor of DNA DSB, a greater understanding of the status of ATM functionality in squamous gynaecological cancers is desirable. Immunofluorescence studies in the 5005T derived primary cells is indicative of prompt formation of pATM foci on induction of DNA DSBs and appropriate resolution of the foci

over an 8 hour time course. This is in keeping with the high nuclear pATM expression seen on the FFPE diagnostic biopsy included on the tissue microarray studied in Chapter 4. It would be interesting to perform similar studies in cells derived from tumours with low nuclear pATM expression to see if this confers impaired pATM foci formation and/or resolution. This work highlights the potential feasibility of such a study.

The evaluation of baseline diagnostic FFPE biopsies for RAD51 foci in geminin positive cells is an intriguing possibility due to the comparative ease of the technique compared to primary cell culture or serial biopsies. This study demonstrates that cervical tissues at baseline demonstrate RAD51 foci in the absence of exogenous agents, and that there was a 2 fold difference in the percentage of geminin positive cells with RAD51 foci in two cervical squamous cancers studied, highlighting potential differential levels of activation. To determine if baseline RAD51 foci could act as a surrogate marker for HRR proficiency and/or predictive of treatment response, further studies would be desirable which could potentially be performed by assembly of a tissue microarray of diagnostic biopsies from patients undergoing treatments such as chemotherapy and radiotherapy.

Chapter 7 Final discussion and future work

Cervical and vulval cancer remain significant health burdens in the UK, and treatments for locally advanced and metastatic tumours can be associated with high morbidity and treatment failure. The mainstay of treatment for these diseases are DNA damaging agents (platinum chemotherapy and radiotherapy) which rely on the creation of overwhelming levels of DNA damage to trigger cell death via apoptosis, whilst at the same time attempting to minimise damage in surrounding tissues to prevent excessive toxicity. It is well recognised that sensitivity to DNA damaging agents is influenced by proficiency of the DNA damage response pathways, as evidenced by the increased sensitivity of BRCA mutation associated cancers to DNA damaging therapies such as platinum chemotherapy (214).

HPV positive tumours have superior treatment outcomes to chemoradiotherapy when compared to HPV negative tumours at sites such as the oropharynx, cervix, and vulva. Currently this phenotype is not well understood, and routine clinical testing of gynaecological cancer HPV status does not take place as it does not currently alter clinical treatment pathways. Evidence from OPSCC cell lines demonstrate impaired repair of DNA DSBs in HPV positive cells compared HPV negative ones in vitro and introducing HPV E7 into HPV negative cells delays DNA repair kinetics (82, 129). These findings occur in a context of generalised upregulation of DDR proteins in the presence of HPV or HR-HPV E6 and E7. The upregulation of DDR genes is thought to benefit the viral amplification phase by the recruitment of DDR proteins to viral replication centres, possibly to facilitate viral replication outside of S phase of the cell cycle (108, 113).

The interaction between HPV and the DDR in gynaecological tumour tissue is not well studied, and the HRR/BRCA/Fanconi anaemia pathway was considered the most promising line of investigation, particularly as this pathway represents the predominant form of DNA DSB repair in replicating cells and is known to be manipulated by HPV oncoproteins and viral DNA replication. Pre-clinical work in mouse models suggests loss of FA pathway activity can accelerate gynaecological malignancies (94) and therefore acquired dysfunction of the FA pathway was hypothesised to play a role in HPV mediated oncogenesis.

This study was successful in developing a bioresource of fixed and fresh tissue for investigations into the HRR/BRCA/FA pathways in cervical and vulval cancers and optimised a protocol for the development of primary cells from these tumours to produce HPV positive early passage cells as a model of HPV mediated oncogenesis.

7.1 ATM in cervical and vulval tumours

This study found that whilst almost all HRR genes studied demonstrated significantly elevated transcription in cervical and vulval tumours compared to cervical normal controls, *ATM* transcription was not elevated with a trend towards decreased *ATM* mRNA levels. This is consistent with prior description of ATM gene methylation and deletion (126). The number of *ATM* mRNA transcripts in tumour samples was not well correlated with levels of activated pATM seen on immunohistochemistry in matched samples, highlighting the difficulties of a transcriptomic approach to infer the activation status of a pathway that is modulated by post-translational modifications in response to DNA damage.

Immunohistochemistry studies demonstrated that HPV mediated cancers express increased nuclear activated ATM, which could represent increased nuclear shuttling of activated ATM to sites of viral replication or genomic damage. One third of cervical tumours with matched adjacent normal tissue, and approximately one fifth of vulval cancers, demonstrated lower overall pATM expression in tumours compared to tissue of origin. This could be due to lower *ATM* transcription, but this is perhaps less likely given the poor correlation seen between the number of *ATM* transcripts and pATM staining scores. It is possible that these tumours exhibit impairment of ATM activation, as it is unlikely that tumour cells have less endogenous DNA damage/DDR activation than normal tissues (although this was not assessed in the tissue samples). It would be of interest to study this possibility further, potentially with immunofluorescence approaches in fixed tissue from these tumours. Tumour pATM levels could be correlated with γH2AX foci as a marker of endogenous DNA damage (γH2AX is phosphorylated by ATR and DNA PK as well as ATM in response to DNA damage).

Further work to prospectively evaluate pATM as a biomarker for treatment response in HPV positive oropharyngeal and gynaecological cancers receiving DNA damaging therapies would also be desirable in view of the findings of this study demonstrating low pATM as an independent predictive factor for improved survival in OPSCC, and similar results have been published in cervical cancer previously (127).

In conclusion, ATM remains a gene of considerable interest in the field of HPV-mediated cancers and this study adds to the current knowledge of the status of this key DDR protein in HPV mediated cancers. This work has not shown evidence impaired activation of ATM in the context of HPV mediated malignancy versus HPV negative disease, but I feel there is scope for further investigation of this with functional studies of pATM foci

formation/resolution in primary cells. It would be interesting to evaluate if cells with lower baseline pATM foci show an appropriate increase in nuclear foci at sites of DNA damage (represented by γH2AX foci) in response to ionising radiation in HPV positive and negative cells.

7.2 FANCD2 in cervical and vulval tumours

This study found that levels of FANCD2 in vulval cancer reflect those in OPSCC cancer, with loss of FANCD2 expression on malignant transformation. Cervical cancer demonstrated the opposite, with a significant increase in nuclear FANCD2, but this does not appear to be related to the increased proportion of HPV positive cancers in the cervical cancer cohort as there was no association found between HPV status and FANCD2 protein expression. It is possible that overall levels of FANCD2 are supressed in vulval and OPSCC cancers via low *FANCD2* transcription, but previous studies did not demonstrate evidence of transcriptional repression in VIN or oral cancer, in fact the opposite with higher FANCD2 expression (106, 155). Unfortunately, a *FANCD2* gene probe was not included in the PanCancer codeset and therefore data in cervical and vulval tumours is not available. It is possible that loss of protein stability and increased rates of FANCD2 degradation could explain the findings of loss of FANCD2 expression in vulval and OPSCC and this would merit further investigation as it could have significant functional consequences of the cellular response to DNA repair.

Unlike studies of ATM where a reliable antibody was available for the activated form of ATM, this study used a FANCD2 antibody which detected FANCD2 in its native, phosphorylated and ubiquitinated state and is therefore perhaps a less reliable surrogate of FA pathway activation. I suggest that further studies to assess FA pathway status in gynaecological tumours would be desirable, and these should evaluate FANCD2 foci formation as a marker of pathway activation. A recent study by Kono et al has performed IF for FANCD2 foci in OPSCC tissue samples and has shown a significant increase in FANCD2 foci in HPV positive but not HPV negative tumours (166), which when compared to the results of this study highlights the potential for discrepancies between total levels of protein and levels of activated protein. A study by Khanal et al using a cell model transfected with HR-HPV E6 and E7 demonstrated increased FANCD2 foci in these circumstances but interestingly these cells also demonstrated increased sensitivity to cisplatin treatment which was not enhanced by FANCD2 knockdown, and therefore postulated to be due to E6/E7 mediated impairment the FA pathway (168). The study showed evidence for two potential mechanisms of FA pathway impairment by HR-HPV E6

and E7. Firstly, evidence of mis-localisation of FANDC2 from areas of DNA DSB (γH2AX foci) in the presence of E6/E7. This is intriguing as raises the possibility that FANCD2 mis-localisation could occur in cells in the presence of E6/E7 in the absence of replicating viral episomes. The second mechanism was that HR-HPV E6 causing prolonged chromatin retention of FANCD2 and impaired FANCD2 deactivation by de-ubiquitination by USP1-UAF1, which is necessary for an ongoing FA pathway DNA repair response (168). Both of these findings could account for impaired FA pathway functionality despite high levels of apparent FANCD2 foci in the presence of in HPV positive tumours. It would be desirable to test these hypotheses in the developed primary cell models by assessing dynamic changes in FANCD2 and γH2AX foci post irradiation. This would allow for determination of appropriate recruitment of FANCD2 to sites of DNA damage, and also to determine if there is impaired resolution of FANCD2 foci suggestive of impaired deactivation.

The FA pathway RNA transcripts evaluated in this study were predominantly upregulated. Closer evaluation demonstrated low levels of transcription of individual FA gene probes (*FANC C, c19orf40, FANCF, FANCG* and *FANCE*) in a small number of tumours which may represent gene hypermethylation as previously reported in FANCF (105). Two cervical cancers samples (5026T, 5023T) demonstrated more than one of these genes with low expression. The functional impact of these findings on DNA repair is not known, but this study highlights a group of genes whose protein expression levels could potentially be evaluated in a larger sample set such as the gynaecological TMAs to determine if low levels occur in a subset of cervical and vulval tumours and if this has any impact on treatment response.

7.3 Cervical and Vulval Transcriptome Analysis

Analysis of DNA repair gene transcription in cervical and vulval tumours demonstrated significant upregulation of genes involved in the DNA repair pathway in tumours when compared to normal tissues, in particular the HRR pathway. It does not appear that HPV mediated oncogenesis exerts transcriptional repression of DDR genes, in fact there appears to be trend towards higher HRR gene expression in HPV positive cancers. Very recently another analysis of the vulval transcriptome analysed with the same PanCancer codeset was published (206), which also demonstrated that HPV positive disease is associated greater levels of expression of DDR genes.

The analysis of RNA expression levels provided novel data in cervical cancer and vulval cancer demonstrating some features that give new insights into therapeutic approaches.

This study identified significantly reduced levels of the *NR4A1* expression in cervical and vulval squamous cancers and it would be interesting to test the hypothesis that this could impair therapy-induced apoptosis and treatment response. I would suggest future immunohistochemical studies would be the best approach to this to determine levels of expression in the separate subcellular compartments within tumours, as it is the cytoplasmic form of the protein that can contribute to stress induced apoptosis.

This analysis demonstrated the dysregulation of cytokines in vulval cancer, specifically with reference *IL1A*, *IL1B* and *IL8* upregulation which promote immune escape and may limit responses to immunotherapy. Conversely the pro-inflammatory *IL24* tumour suppressor gene and the *IL-12RB2* receptor gene were also upregulated in vulval tumours. It would be interesting to characterise individual tumour cytokine signatures in vulval cancers treated with immunotherapies in the future in the context of clinical trials to see if they are predictive of response.

In HPV negative disease, the transcriptomic profile of the Hedgehog pathway was indicative of activation compared to HPV positive tumours. This corroborates a recent publication by Yap et al demonstrating aberrant activation of Hedgehog signalling in vulval squamous cancers (215). This can be considered a potential therapeutic target for vulval cancers, and there is the potential for trials of hedgehog inhibitors that are in clinical development (216).

7.4 COMICE clinical trial

A multi-centre randomised controlled phase II trial is underway in the UK recruiting patients with advanced or recurrent cervical cancer following an initial response to platinum chemotherapy. Patients are randomised in a 1:1 fashion between placebo maintenance treatment (standard of care arm) and oral maintenance therapy with cediranib (a tyrosine kinase inhibitor of VEGFR1-3) and olaparib (a PARP inhibitor). The study protocol is outlined in a submitted publication (Appendix D).

Cervical cancers are responsive to anti-angiogenic therapy as evaluated in the GOG 240 study demonstrating overall survival benefit with bevacizumab with first line chemotherapy. At present bevacizumab is administered for up to 6 cycles with chemotherapy and there is no maintenance therapy in this disease group in the UK. Cediranib was previously investigated in the CIRCCa study (217) alongside first line chemotherapy in advanced and recurrent cervical cancer and then continued in a

maintenance fashion until disease progression or unacceptable toxicity. This demonstrated PFS improvement of 1.4 months, but no OS data has been published and the superior outcomes with bevacizumab including 3.7 OS benefit have led to this becoming the UK standard of care alongside chemotherapy.

The COMICE study aims to determine if further improvement in clinical outcomes can be achieved with oral maintenance anti-angiogenic therapy following chemotherapy with bevacizumab in advanced cervical cancer. The addition of olaparib to the maintenance regime is intended to give enhanced therapeutic efficacy due to synergistic activity between the two agents in a combination that is also under investigation in ovarian (ICON 9 trial) and endometrial cancer (COPELIA trial).

PARP inhibitors trap PARP1 and PARP2 at sites of single strand DNA breaks, inhibiting repair of SSBs and causing replication fork collapse and DNA DSBs in replicating cells (218). This can be catastrophic for tumour cells less able to effectively repair DNA DSBs and this class of drug is now widely used in tumours with exhibiting homologous recombination repair deficiency (HRD). In ovarian cancers PARP inhibitors are used as a first line maintenance monotherapy treatment (219, 220) or in combination with maintenance angiogenic therapy (bevacizumab) (221) after response to platinum chemotherapy. The most benefit is seen in tumours exhibiting BRCA mutations or high HRD scores based on companion diagnostic tests such as myriad MyChoice®. However, there is still benefit in patients without these biomarkers in terms of PFS, and to some degree this probably reflects the imperfect nature of interpreting DDR proficiency in fixed tissue (220).

Given the increasing body of evidence to suggest that HPV mediated cancers have impaired DNA DSB kinetics, there is rationale for assessing the efficacy of PARP inhibition in this group of tumours. The use of a rational combination with an anti-angiogenic agent in this tumour groups is desirable given the already known activity of anti-angiogenic agents in cervical cancers. The combination of olaparib and cediranib gave improved response rates and progression free survival compared to olaparib alone in a phase II study of maintenance treatment in ovarian cancer (222). There is evidence that cediranib therapy sensitises tumours cells to PARP inhibition by the down regulation of BRCA 1, BRCA 2 and RAD51 and hypoxia has also been shown to down regulate FA protein expression (223-226).

During the COMICE clinical trial development process I designed a translational research program to evaluate baseline diagnostic FFPE cervical cancer samples (and any subsequent biopsy specimens sampled during routine clinical care) for biomarkers relevant to response to olaparib and cediranib. Serial plasma samples taken at key timepoints during treatment have also been collected to monitor potential circulating factors predictive of anti-angiogenic response. The preliminary protocol is found in Appendix D. It was anticipated that the work contained in this thesis would inform those future studies.

The findings of this thesis are useful in prioritising the translational research program for COMICE. On the basis of the findings from the Nanostring analysis, I don't feel that extensive transcriptomic profiling is likely to give a good representation of DDR functionality in cervical cancers as a predictive biomarker for PARP inhibitor response. The PanCancer codeset was selected for the inclusion of key angiogenic factors (VEGFA, VEGFC, VEGFD, VEGFR1, HIF1), but I feel a better approach would be targeted RT-PCR analysis of transcriptional activity of selected angiogenic factors, or indeed evaluation of expression of angiogenic factors in tumour tissue by IHC on a TMA.

HRD assays are now used in routine use to evaluate tumour HRR status in ovarian cancers and use of this (or a similar assay as initially proposed to evaluate genomic scarring by loss of heterozygosity) may give some insight into the level of HRD in tumour samples. As the level of HRD in HPV mediated cancer is perhaps not anticipated to be so profound as in patients with DNA repair gene mutations, such assays may not be predictive of response as demonstrated in the subset of ovarian cancer patients without BRCA mutations and with low MyChoice® HRD scores, who still respond in a more modest fashion to niraparib therapy (220).

I would therefore suggest that the main focus a translational research program for the COMICE study would be utilising fixed tissue (potentially on a tissue microarray) to analyse the activation status of the HRR/BRCA/FA pathway by immunofluorescence studies using the same technique as the pilot studies in 6.6.3. The clinical study will also give a large resource of fixed tissue to further explore the status of NR4A1 in cervical cancer. To accompany these studies, I think utilisation of the developed primary cell resources to evaluate the effect of hypoxia on the DDR would be of interest by growing cell in hypoxic conditions, with or without olaparib treatment to further understand potential synergistic mechanisms between the two agents in tumour cells.

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Chapter 9. Appendices

Appendix A Supplementary Protocols

1. Isopentane freezing Protocol for Gynaecological tissue collection

Always process adjacent normal specimens before any tumour samples.

1. Before sample collection
    a. label cryovials
    b. **in fume cupboard** place 30mls polypropylene pot on dry ice in polystyrene container
    c. pour 20 mls of isopentane into pot and add a small amount of liquid nitrogen/dry ice to cool isopentane
2. On return with clinical samples use sterile forceps place tissue samples into tube of isopentane for 5-10 seconds
3. Remove frozen tissue and place in cryovial
4. Transport to CRC on dry ice
5. Store at -80^{0}C until used

2. Tissue collection protocol

Tumour tissue procurement to allow establishment of primary cell cultures for use in Human papillomavirus-mediated cancer research

Version 1.2 03/10/16

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Study Team

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Clinical Queries

Clinical queries should be directed to Joseph Sacco who will direct the query to the appropriate person.

Sponsor

The University of Liverpool is the main research Sponsor for this Study. For further information regarding the sponsorship conditions, please contact:

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STUDY SUMMARY

This protocol describes the 'Tumour tissue procurement to allow establishment of primary cell cultures for use in Human papillomavirus-mediated cancer research' study and provides information about procedures for entering participants. Every care was taken in its drafting, but corrections or amendments may be necessary. These will be circulated to investigators in the Study. Problems relating to this Study should be referred, in the first instance, to the Chief Investigator.

This study will adhere to the principles outlined in the NHS Research Governance Framework for Health and Social Care (2nd edition). It will be conducted incompliance with the protocol, the Data Protection Act and other regulatory requirements as appropriate.

GLOSSARYOFABBREVIATIONS

HPV	Human papillomavirus

KEYWORDS

Human papillomavirus

Cervical Cancer

Vulval Cancer

Vaginal Cancer

TITLE Tumour tissue procurement to allow establishment of primary cell cultures for use in Human papillomavirus-mediated cancer research

AIMS To establish a model for HPV-positive Gynaecological cancer research

POPULATION ELIGIBILITY Patients with gynaecological squamous cell cancers (cervix, vaginal, vulval)

DURATION 7 years

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1.INTRODUCTION

1.1 BACKGROUND

High-risk types of Human papillomavirus (HPV) are annually implicated in the aetiology of at least 600,000 malignancies worldwide including cervical, vulval, vaginal, anal and oropharyngeal cancer. These tumours cause significant morbidity and mortality and unified national and international calls have been directed towards improving the understanding of HPV-positive malignancy across tumour sites.
Within our region there is an established tissue resource developed under the governance of the Merseyside Head & Neck Oncology Research Group (MHNORG) for primary cell lines from HPV-positive Oropharyngeal Squamous Cell Carcinomas (OPSCC). This study aims to develop a similar resource for the study of gynaecological HPV-positive cancers within the region to gain further insight into these virally mediated malignancies and to allow comparative studies between the two models of investigation.

1.2 RATIONALE FOR CURRENT STUDY

A particularly profound paradox in HPV-related malignancy is currently unexplained; how does an apparently identical, virally mediated oncogenic process confer contrasting disease outcomes in different tumour subsites. This project aims to develop a disease model upon which to study this contradiction.
As such, the project offers an opportunity to inform further research and, conceivably, clinical management of HPV-mediated disease at several disease sites. The proposed early passage primary cell culture-derived model offers significant advantages over established, later-passage and commercially available secondary cell lines. These early passage primary cells are more representative of the originating tissue, in contrast to the protracted in vitro environmental influences and multiple passages in secondary cell culture that are unfavorably influenced by selective pressures, genetic drift and loss of cellular heterogeneity. The availability of such a primary culture model, capable of closely replicating tumour biology of HPV-positive gynaecological malignancy is not currently available through either collaborative or commercial channels.

2. STUDY OBJECTIVES

This project intends to establish an HPV-positive gynaecological malignancy primary cell culture resource with the ambition of serving two distinct aims.
Firstly, once established, this resource will permit a shift within our cellular oncology research, away from a reliance on secondary cell lines towards a model more representative of the original tumour, yet still permitting functional workflows.

Secondly, this project is designed to run in parallel to a similar project, already established under the governance of the Merseyside Head & Neck Oncology Research Group (MHNORG), which is developing an equivalent primary cell resource of HPV-positive Oropharyngeal Squamous Cell Carcinomas (OPSCC). Clearly, the potential for comparative analysis between these resources is substantial and offers a tangible research opportunity that, to our knowledge, is not available elsewhere.
In keeping with these aims, we will undertake full characterisation of the resultant primary cultures, the primary tumour samples, and adjacent normal tissues (and their related primary cultures), such that any variation from the parent primary tumour is established and documented. Further, we intend to undertake necessary initial functional work to establish the reliance (or otherwise) of the primary cultures on viral oncogenes through knockdown of HPV16 E6 & E7 expression with shRNA. Matched venous blood samples will also be stored for future research (analysis of blood-based biomarkers of tumour phenotype, relevant control in genomic assays, circulating tumour cell analysis). Once characterised, we plan to study the established primary cell cultures in an organotypic model as previously described (1, 2). It is also possible that during the passaging of primary cell cultures that an immortalised secondary cell line may develop, which may be used for further research.

To increase the sample number of gynaecological squamous cell tumours studied as part of this project we also plan to recruit participants previously diagnosed with cervical or vulval cancer for consent to access their archived formalin-fixed paraffin embedded (FFPE) tissue for research purposes, and to utilise frozen gynaecological squamous cell cancer specimens and matched plasma/cellular fraction blood fractions currently held in the Liverpool Women's Research Tissue Bank. These will be analysed for DNA, RNA and protein to validate any molecular aberrations identified in the primary cell cultures.

3. STUDY DESIGN

This study aims to collect fresh and frozen tissue (tumour and adjacent normal) and a venous blood sample from patients undergoing a planned surgical procedure for management of a squamous gynaecological malignancy. Many of these patients will be having tissue removed as part of their treatment, and hence tissue sampling presents no additional risk to participants. Participants will also be asked to consent pre-operatively for dedicated research biopsies to be taken in the event that tissue removal is not otherwise needed at time of surgery (such as during examination under anaesthesia). Research biopsies will only be taken if they can be performed without any detrimental effect to the patients care, and it is anticipated to be a low risk procedure given the largely exophytic nature of the tumours in question.
To provide a validation cohort to support findings in fresh tissue ad primary cell models, we also plan to recruit participants who have previously undergone a diagnostic biopsy or surgical procedure for cervical, vulval or vaginal cancer for consent to access archival tissue for evaluation.

We anticipate this study will be open for a 7 year period, recruiting 24 participants for fresh tissue and blood donation, and 100 participants for archival fixed tissue evaluation.

3.1 STUDY OUTCOME MEASURES

There will be no patient-related outcome measures from this study.

4. PARTICIPANT ENTRY

4.1 PRE-REGISTRATION EVALUATIONS

Before a participant can enter the study the patient has to be evaluated by the treating clinician and to be identified as a patient from whom fresh tissue collection would not adversely affect the diagnostic pathological margins of tissue taken for histopathological evaluation, or compromise the treatment procedure.

4.2 INCLUSION CRITERIA

The principal inclusion criteria will be a diagnosis of a gynaecological squamous cell malignancy (cervical, vulval, vaginal) having previously undergone a diagnostic biopsy or surgical procedure or requiring a further surgical procedure as part of routine care.

4.3 EXCLUSION CRITERIA

Patients under 18 will be excluded from this study.

Patients who cannot sufficiently understand the English language to provide informed consent will be excluded if a suitable interpreter is not available in the clinical setting.

4.4 WITHDRAWAL CRITERIA

There are no withdrawal criteria for this study. Patients may withdraw from the study up to 5 years after recruitment should they wish. After this period, it will no longer be possible to identify and destroy the tissue samples of individual participants due to the deletion any of personal information linked to study samples.

5. ADVERSE EVENTS

5.1 DEFINITIONS

There are no anticipated adverse events from taking part in this study.

6. ASSESSMENT AND FOLLOW-UP

There will be no planned direct contact between the participant and the research team following tissue collection. Participants will be asked to consent to a member of the research team accessing their case notes for clinical outcome data for our research database. This database will provide anonymised information regarding clinical disease outcomes to correlate with research findings.

7. STATISTICS AND DATA ANALYSIS

There is no planned statistical analysis as part of this study.

8. REGULATORY ISSUES

8.1 ETHICS APPROVAL

The Chief Investigator will obtain approval from the Adult North West Research Ethics Committee prior to commencement of study recruitment. The study must be submitted for Site Specific Assessment (SSA) at each participating NHS Trust. The Chief Investigator will require a copy of the Trust R&D approval letter before accepting participants into the study. The study will be conducted in accordance with the recommendations for physicians involved in research on human subjects adopted by the 18th World Medical Assembly, Helsinki 1964, and later revisions.

8.2 CONSENT

Consent to enter the study must be sought from each participant only after a full explanation has been given, an information leaflet offered, and time allowed for consideration. Signed participant consent should be obtained. The right of the participant to refuse to participate without giving reasons must be respected. All participants are free to withdraw from the study up to 5 years following recruitment without giving reasons and without prejudicing further treatment.

8.3 CONFIDENTIALITY

The Chief Investigator will preserve the confidentiality of participants taking part in the study and is registered under the Data Protection Act.

8.4 INDEMNITY

The University of Liverpool holds Indemnity and insurance cover with Marsh UK LTD, which apply to this study.

8.5 SPONSOR

The University of Liverpool will act as the main Sponsor for this study. Delegated responsibilities will be assigned to the NHS trusts taking part in this study.

8.6 FUNDING

Clatterbridge Cancer Centre Charitable Funds are funding this study.

8.7 AUDIT

The study may be subject to inspection and audit by the University of Liverpool under their remit as sponsor and other regulatory bodies to ensure adherence to GCP and the NHS Research Governance Framework for Health and Social Care (2nd edition).

9 STUDY MANAGEMENT

The day-to-day management of the study will be coordinate through The North West Cancer Research Centre, University of Liverpool.

10. END OF STUDY

The study will recruit participants for up to 2 years, with a period of clinical data follow up for 5 years. The study will therefore end at 7 years, at which time a review of the feasibility and utility of the project will take place.

11. ARCHIVING

Data and all appropriate documentation (excluding patient identifiable data) will be stored for a minimum of 5 years after the completion of the study.

12. PUBLICATION POLICY

Scientific research undertaken in relation to this project will be published in peer-reviewed journals and presented at academic meetings by the research team.

13. REFERENCES

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14. APPENDICES

i. Patient information Sheet
ii. Patient Consent form
iii. GP letter
iv. SOP for tissue collection at Liverpool Womens Hospital
v. SOP for tissue collection at Clatterbridge Cancer Centre

Appendix i – P.I.S

Establishing a model for HPV-associated Gynaecological Cancer Research

Patient Information Leaflet

Collection and storage of tissue and blood for research

Version 1.2 03/10/2016

Consent to storage and use of tissue for research

Contact Information for Study

Dr Joseph Sacco

University of Liverpool

Department of Molecular and
Clinical Cancer Medicine

5th Floor, UCD building

Royal Liverpool University Hospital

L69 3GA

L2 9TA

We would like to invite you to take part in clinical research by donating tissue removed during a biopsy or surgical procedure to be used for research into Human papillomavirus (HPV) -associated gynaecological cancers. Before you decide whether to donate tissue, you need to understand why we are requesting tissue and what collection of tissue will involve. Please take time to read the following information carefully. It contains important information about the study and will help you to decide whether you would like to take part. If you wish, you can take it away with you to discuss it with family or friends. Please ask your doctor if there is anything that is not clear, or if you would like more information.

What is the purpose of the study?

Human papillomavirus infection is very common, and most women are exposed to the virus at some point in their lives without experiencing any symptoms. We know that certain gynaecological cancers in women are associated with 'high-risk' HPV infection, but we don't know why some women develop cancer whilst most women exposed to the virus do not encounter any problems related to the infection.

This study aims to analyse cancer cells from sites where HPV infection could have a role in the development of cancer (cervix, vagina, vulva) to gain a greater understanding of factors which lead to cancer at these sites. This study will not affect your treatment in any way.

Why have I been chosen?

You have been asked to participate in this study because you have had, or are going to have, a procedure involving an area which may potentially be affected by the HPV virus.

What will happen to me if I take part?

If you have already had a biopsy or surgical procedure, we will ask for your consent to take small samples of the tissue you had removed (approximately the size of a grain of rice) to be stored for research purposes.

If you decide to take part in this study and are due to undergo a biopsy or surgical procedure we will ask for your consent to take small tissue samples (approximately the size of a grain of rice) from the area of cancer and from healthy tissue next to the cancer in the biopsy, which will then be stored for research purposes. The tissue

will be taken during your operation so you will be asleep under a general anaesthetic and will not experience any additional discomfort.

If we take some tissue during your surgical procedure, we will also ask you for consent to take a small amount (approximately 4 teaspoons worth) of blood for research use. This extra blood sample will be taken by the anaesthetist at the time of your operation.

As part of this study we would like your consent to access your health records. These will be reviewed by a member of the research team on an annual basis in order to update information on our research database. All information will be treated with the strictest confidence and held securely within the University of Liverpool. This information is essential to help researchers understand the course of your disease and relate what is found in the laboratory to what has happened following your treatment. Your name, address and any other personal data will be removed before any information is given to research groups, so you will not be identifiable to the researchers.

What are the advantages and disadvantages of taking part?

The samples taken for storage and research are small and will be taken during or after your surgery once the necessary diagnostic samples and/or tests have been performed. The results of research carried out using your tissue, blood and/or biological samples, and those of others, may help in the future discovery of new drugs and treatments for women with gynaecological cancer. There will be no direct benefit to yourself and you will not be identifiable to the research team.

If your doctors have already removed tissue or are planning to remove tissue as part of your surgical procedure, there are no additional risks if you choose to participate. The risks associated with surgery will be explained separately by the medical team as part of your treatment.

If your doctors are not already planning to remove tissue as part of your surgery, we may ask you if a biopsy can be taken during a planned procedure for use in research. This will be performed by the surgeon undertaking the planned treatment, while you are under anaesthesia, and will not impact on the planned procedure. The removal of a small amount of tissue is associated with a small increased risk of bleeding and infection. In many cases antibiotics are given routinely as part of your surgical procedure, which acts to reduce the risk of infection. If a blood sample is taken, occasionally, this may require an additional needle entry site whilst you are under general anaesthetic. There is a small chance that you may experience some bruising at the site.

Do I have to take part?

No. The choice to take part in this study is up to you. If you do decide to consent to donate tissue then you can keep this information document and you will be asked to sign a consent form. Even if you do decide to donate tissue, you are free to change your mind at any time and without saying why. Whatever your decision it will not affect the standard of care that you receive.

What will happen if I change my mind?

If you do decide to take part, you are still free to change your mind. You have the right to withdraw your consent to store your tissue, blood and/or biological samples without giving a reason. This can be done up to 5 years after you consent to study participation. After this period of time, we will no longer be able to identify your samples which will have been anonymised for research use. If you do withdraw, then it will not affect in anyway the treatment that you are receiving. You can withdraw your consent by contacting the research team by writing to:

Dr Joseph Sacco

University of Liverpool

Department of Molecular and Clinical Cancer Medicine

5th Floor, UCD building

Royal Liverpool University Hospital

L69 3GA

The tissue, blood and/or biological samples stored by the University, along with any information held about you, will be destroyed and a letter of confirmation will be sent to you. If you change your mind a long time after the samples were donated, then some research may have already taken place on your samples. It would not be possible to recall samples and information once they have been used, but we would ensure that no further research work will be undertaken on your tissue, blood and/or biological samples.

What if anything goes wrong?

We do not anticipate that participation in this study will lead to any harm. If you have any concerns or complaints, please ask to speak with the researchers who will do their best to answer your questions, or directly contact the research team by writing to:

Dr Joseph Sacco

University of Liverpool

Department of Molecular and Clinical Cancer Medicine

5th Floor, UCD building

Royal Liverpool University Hospital

L69 3GA

If you remain unhappy and wish to complain formally, you can do this through the normal NHS Complaints Procedure. Details can be obtained from the hospital.

The sponsorship team at the University of Liverpool can provide independent assistance with a complaint. Please contact:

Mr Alex Astor
Research Support Office
2nd Floor Block D Waterhouse Building
3 Brownlow Street
Liverpool
L69 3GL
Tel: 0151 794 8739

What will happen to my tissue or blood?

Academic research groups will use your gift of tissue and blood to understand the causes of HPV-associated gynaecological cancer and help to develop the best treatments and care for women in the future. If you are donating tissue at the time of your surgical procedure, part of this research will involve growing live cells taken from your tissue donation in the laboratory to see how they behave in response to treatments. Sometimes this process brings about changes in the cells which encourage indefinite growth. In all cases research studies will be ethically approved and monitored. Your tissue will not be used for transplantation or reproductive cloning. Nor will the tissue be used for non-medical or non-scientific purposes. Live cells derived from your tissue donation may be used in future studies and shared with other researchers. Whilst the University will not sell your tissues for profit to other researchers, future research may lead to commercial gain which you will not benefit financially from.

When we store samples, we will use some of them to obtain genetic material (DNA and RNA) and protein. We are asking you to allow us to obtain DNA, RNA and protein so that this can also be made available to research groups. We will not use DNA, RNA or protein samples for any purpose other than research and the research team will not be able to identify you in any way.

With your consent at the end of this study any remaining tissue or blood donated by you would be placed in a research tissue bank to allow further research to be carried out with ethical approval. In all cases, you will be anonymous to the researcher.

What if Researchers find new information about my health?

We do not anticipate any discoveries during this research which will be relevant to your care and treatment.

Will anybody make a profit from my tissue, blood and/or biological samples?

You are asked to donate your tissue for research and will not receive a financial reward either now or in the future. Similarly, the University will not sell your tissue for profit to other researchers.

What will happen to the results of the research study?

It is anticipated that this research will take several years to complete. Results will be published when appropriate in scientific papers and magazines and at scientific meetings. You will not be able to be identified if research using your tissue, blood and/or biological samples is published in any scientific papers.

Will my taking part in this study be kept confidential?

All information that is collected related to your medical condition will be kept strictly confidential. Your name, address and other personal information will be removed before any information is released to researchers using your tissue, blood and/or biological samples. You will not be able to be identified by the researcher. We will ask you for permission to inform your GP of your participation in the study.

Who has reviewed this study?

This study has been reviewed and approved by the RES Committee Northwest – Liverpool East.

Appendix ii – Consent Form

Establishing a model for HPV-associated Gynaecological Cancer Research Consent Form
Collection and Storage of Tissue and blood for Research

If you agree to take part, please initial each box and sign and date this form

1. I have read and understood the information leaflet (Version…….) on the above research project and have been given a copy to keep. I have had the opportunity to ask questions about the project and understand the benefits and risks of donating. ☐
2. I agree to give samples of my tissue from any procedures that are undertaken as part of my treatment. ☐
3. **(Optional)** I agree for an extra biopsy to be performed during my planned surgical procedure (for research) ☐
4. I agree to give a sample of my blood for research use ☐
5. I understand how the samples will be collected, that giving a sample is voluntary and that I am free to withdraw my approval for use of the samples at any time without giving a reason and without my care or legal rights being affected. ☐
6. I agree that the University of Liverpool will become custodian of this tissue and/or blood for use in regulated research projects. ☐
7. I agree that research staff can collect and store information on an ongoing basis for updating the research database from my health records that this information may be viewed by regulatory authorities. I understand that information about me will be treated confidentially and stored securely. I give permission for you to contact my GP regarding my participation in this study. ☐
8. I agree to allow any unused tissue from this study to be placed into a tissue bank and that this may be used for future projects on gynaecological cancer with ethical committee approval. ☐
9. I understand that any information given to research groups will be anonymised and my identity will be protected. I agree to samples being sent to research groups based in the UK. ☐
10. I agree that it may be appropriate for genetic assessment of the samples to be carried out to determine whether genetic makeup has any influence on my condition. ☐
11. I understand that I will not benefit financially if research using my samples leads to new treatments or medical tests. The University of Liverpool will hold intellectual property rights. ☐

Patient Statement I agree to give tissue and blood for use in medical and scientific research.

Signed…….Print Name……Date……

Clinical/Research Practitioner Statement I have explained the request for samples for research purposes and have answered such questions as the patient has asked.

Signed…….Print Name……Date……
Please complete the following patient details:

Hospital Unit Number……NHS Number……

Appendix iii – GP letter

North West Cancer Research Centre
Department of Clinical Cancer Medicine
University of Liverpool
200 London Road
Liverpool
L2 9TA

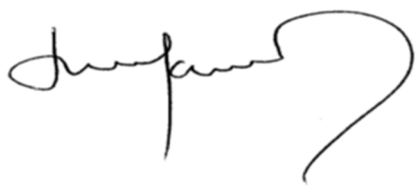
Dear Doctor

Re

I am writing to inform you that the above patient has consented to participate in a study being conducted at Liverpool Women's NHS Foundation Trust and the University of Liverpool. The aim of the study is to establish a model for HPV-associated Gynaecological Cancer Research using samples of tissue and blood.

If you require any further information please do not hesitate to contact me.

Kind Regards



Dr Joseph Sacco
Senior Clinical Lecturer and Consultant in Medical Oncology

Appendix iv

SOP for Gynaecological tissue collection at Liverpool Women's Hospital (LWH)

1. Obtain written consent prior to operation and assign patient study number (5xxx) with associated sample numbers (8xxx) on database.

2. Label tubes and sample collection sheet to correspond with database. Place collection sheet with its corresponding tubes into a plastic bag. Complete participant data collection sheet.

3. Prepare for tissue collection and processing in ITM laboratory at LWH

   3.1 pre-cool isopentane as per isopentane freezing protocol

   3.2 pre-warm transport medium in water bath to 37^{o}C

4. At LWH anaesthetist (or other competent clinician) to collect 10ml citrated blood during pre-operative venous cannulation or after anaesthesia in theatre.

   4.1 Label blood tube with patient study number and sample number; ensure that all other patient identification is removed from tube.

5. Collect tissue samples from tumour and uninvolved margin. Ideally collect 3 sections, this may represent a single larger sample subsequently sectioned into 3, each being at least 5mm^3.

   5.1 Place 1 tumour and 1 adjacent normal tissue sample into pre-labelled tubes and keep on dry ice for transport.

   5.2 Place 1 tumour and 1 adjacent normal tissue sample into pre-labelled tubes containing transport medium, place in secure container for transport.

   5.3 Place 1 tumour and 1 adjacent normal tissue sample in pre-labelled tubes containing RNA later (if sufficient tissue available), place in secure container for transport.

6. Transport biological samples to ITM laboratory at LWH

   6.1 Centrifuge blood sample for 10 minutes at 1800rcf at 4^{o}C within 4 hours of collection. In class II tissue culture facility, remove plasma into five separate, pre-labelled tubes in 200microL aliquots and store any residual plasma in 1mL aliquots. Store buffy coat in 500microL aliquots.

   6.2 Remove tissue sample from empty tube and freeze with isopentane within 1 hour of sample collection. Place in pre-labelled cryovial.

   6.3 Place frozen tissue on dry ice for transport.

6.4 Place tissue in RNA, plasma and buffy coat aliquots and tissue in transport medium in a secure container at room temperature for transport.

7. Transport all biological specimens to North West Cancer Research Centre

7.1 On arrival at North West Cancer Research Centre place frozen tissue, plasma and buffy coat in dedicated research -80^{o}C freezer

7.2 Process tissue in transport medium as per *Primary Cell culture protocol for gynaecological tissue*

7.3 Store RNA later specimen at 4^{o}C for up to 3 months, at which time the RNA must be decanted and the tissue stored at -80^{o}C

8. Open the offline patient identification file

8.1 Check that samples have the correct patient number associated with them

8.2 Fill in New patient data using data from participant data collection sheet

9. Open the coded table in the main database,

9.1Add the new samples and fill in details as appropriate

9.2 Open samples table in the main database; cross check study numbers and sample numbers; fill in dates of sample, sample details and storage site

10. Place Consent forms/sample collection forms/participant data sheet in the locked collection file.

11. All samples to be stored and analysed at North West Cancer Research Centre.

Appendix v

SOP for Gynaecological tissue collection at Clatterbridge Cancer Centre (CCC)

1. Obtain written consent prior to operation and assign patient study number (5xxx) with associated sample numbers (8xxx) on database.

2. Label tubes and sample collection sheet to correspond with database. Place collection sheet with its corresponding tubes into a plastic bag. Complete participant data collection sheet.

3. Prepare for tissue collection and processing in laboratory at North West Cancer Research Centre

   3.1 Pre-cool isopentane as per isopentane freezing protocol

   3.2 Pre-warm transport medium in water bath to 37^{o}C

4. At CCC Anaesthetist (or other competent clinician) to collect 10ml citrated blood during pre-operative venous cannulation or after anaesthesia in theatre

   4.1 Label blood tube with patient study number and sample number; ensure that all other patient identification is removed from tube.

5. Collect tissue samples from tumour and uninvolved margin. Ideally collect 3 sections, this may represent a single larger sample subsequently sectioned into 3, each being at least 5mm^{3}.

   5.1 Place 1 tumour and 1 adjacent normal tissue sample into pre-labelled tubes and keep on dry ice for transport.

   5.2 Place 1 tumour and 1 adjacent normal tissue sample into pre-labelled tubes containing transport medium, place in secure container for transport.

   5.3 Place 1 tumour and 1 adjacent normal tissue sample in pre-labelled tubes containing RNA later (if sufficient tissue available), place in secure container for transport.

6. Transport biological samples to laboratory at North West Cancer Research Centre.

   6.1 Centrifuge blood sample for 10 minutes at 1800rcf at 4^{o}C within 4 hours of collection. In class II tissue culture facility, remove plasma into five separate, pre-labelled tubes in 200microL aliquots and store any residual plasma in 1mL aliquots. Store buffy coat in 500microL aliquots. Place frozen tissue, plasma and buffy coat in dedicated research -80^{o}C freezer

   6.2 Remove tissue sample from empty tube and freeze with isopentane within 1 hour of sample collection. Place in pre-labelled cryovial and store in -80 ^{o}C freezer.

6.3 Process tissue in transport medium as per *Primary Cell culture protocol for gynaecological tissue*

6.4 Store RNA later specimen at 4^{o}C for up to 3 months, at which time the RNA must be decanted and the tissue stored at -80^{o}C

7. Open the offline patient identification file

7.1 Check that samples have the correct patient number associated with them

7.2 Fill in New patient data using data from participant data collection sheet

8. Open the coded table in the main database,

8.1 Add the new samples and fill in details as appropriate

8.2 Open samples table in the main database; cross check study numbers and sample numbers; fill in dates of sample, sample details and storage site

9. Place Consent forms/sample collection forms/participant data sheet in the locked collection file.

10. All samples to be stored and analysed at North West Cancer Research Centre.

3. Primary Cell Culture Protocols for Gynaecological Tissue

Materials

- Media
    - DMEM; F12 Ham Nutrient Mixture (Sigma)
    - RPMI (Sigma)
    - Keratinocyte serum free media with supplements (SFKM, Life Technologies)
    - Glasgow modified eagle's medium (GMEM, Sigma)

- Supplements
    - L- glutamine
    - Fetal Bovine Serum (FBS) South American (Life Technologies)
    - Epidermal growth factor (EGF, Sigma)
    - Hydrocortisone (Sigma)
    - Cholera toxin (Sigma)
    - Penicillin-Streptomycin, sterile-filtered (Sigma)

- Trypsin-EDTA solution 1× (Sigma)
- Phosphate-Buffered Saline
- Recovery™ Cell Culture Freezing Medium (Life Technologies)
- 2% Virkon®
- Sterile falcon tubes
- Scalpels (Swann-Morton size 21)
- CellBind 6 well plates (Corning)
- TC treated dish, Easy-grip 35mm
- 5ml, 10ml and 25ml pipettes
- Pastettes – single wrapped, sterile
- 25 cm3 vented tissue culture flask (Falcon)
- Cryovials (blue capped internal thread)
- Cell scrapers
- 10% buffered formalin (4% PFA)
- Sterile coverslips

Tissue collection

Tissue is collected directly from theatre. Tissue is transported in transport media.

- DMEM (Gibco)
- 10% FBS
- 1% L-Glutamine
- 2 x pen/strep

To theatre bring:

- 1 universal for Normal tissue
- 1 universal for Tumour tissue

- If applicable, 1 universal for Node tissue
- Each with 10ml of suitable transport medium
- Zip lock bag to store universals

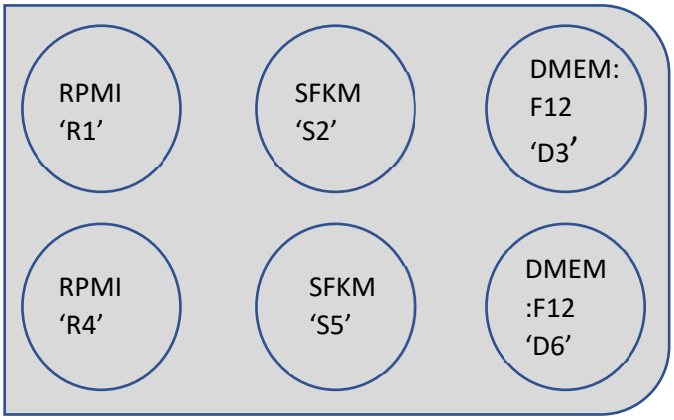
Keep Transport medium warmed to 37° until you need to leave for theatre (and some for on return).

Tissue preparation

- On returning to the lab, transfer the tissue to fresh transport medium in the hood using a pastette and wash on a roller for 1hr, changing to fresh medium a further 2 times (3 washes in total) within the hour.
- Process normal, then tumour.
- Remove tissue from the wash medium and transfer to a sterile well, adding 1-2 drops of wash medium.
- Trim off visible blood vessels and fat deposits using sterile scalpels (Swann-Morton size 21).

Primary Cell Culture Protocol 1

- Transfer half of the trimmed tissue to a sterile CellBind 6-well plate
- Dissect tissue into small pieces (1-2mm^3) in a 'chopping' motion – don't saw or scrape tissue – using the 2 scalpels from previously.
- Add a few more drops of wash medium to the 'mushed' tissue and, using the tip of a pastette, seed the other 5 wells with roughly the same amount of tissue in each. Ensure there is just a 'film' of medium covering each well. Break up any clumped tissue.
- Incubate at 37° for 30mins-1hr
- Gently add suitable medium (see below) (which is at 37°) drop wise to each well until tissue is just covered. Run the medium down the sides of the well to avoid dislodging the adhered tissue.
- Incubate at 37° overnight. Leave tissue undisturbed for the duration of the next day.
- On the 2nd day after seeding the cells, gently top up the medium in each well with an additional 1ml
- On the 4th day combine the media from the 2 similar wells and transfer to a T25 flask. These are labelled as passage 1. Leave this at just ~4ml for 24hours before topping up with additional medium. Top up the wells in the plate with 3ml fresh medium.



Primary Cell Culture Protocol 2

- Rinse a TC coated petri dish with FBS and aspirate off.
- Transfer biopsy to coated dish and dissect tissue into small pieces (1-2mm^3) in a 'chopping' motion, adding more drops of FBS if required to avoid tissue drying out.
- Place lid on dish and invert gently - place in incubator at 37°C in 5% CO2 for 1-2hrs.
- Aspirate any residual FCS
- Premix feeder cells (5x 10^5) with EGF negative media (GMEM, 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin, 250 µl hydrocortisone, 500µl cholera toxin)
- Gently add EGF negative media and feeders to explant plate, avoiding detachment of tissue and incubate at 37°C in 5% CO2 for 3-4 days, topping up media if drying out.
- Day 3 or 4 change the media to EGF positive media (1% EGF), adding more irradiated feeders if necessary.

Cell passaging

- Once there are explants coming from the tissue these can be trypsinised and transferred to a T12.5 flask
- Don't combine colonies from separate wells into 1 flask.
- To trypsinise well or dish
    - Wash wells/dishes with PBS (if feeder cells present remove as many as possible by squirting or brief trypsinisation)
    - Add 1-2ml trypsin and incubate at 37°C for 3mins
    - Using a cell scraper, gently scrape the areas where there are colonies (if mixed cell populations use the scraper on just the desired colonies)
    - Take up the trypsin and cells and add to sterile falcon tube with 10ml DMEM:F12 w/10% FBS or EGF negative GMEM depending on protocol
    - Spin at 1200rpm for 5mins
    - Discard medium
    - Resuspend pellet in 3ml of the appropriate medium (EGF negative if GMEM) and transfer desired number of cells to T12.5 flask (count with haemocytometer)
    - Add 2 x 10^6 feeder cells if required
    - After 48 hours can replace EGF negative media with GFR positive media

- This first trypsinisation is passage 1.

- Wash well/dish with PBS and top up with fresh medium if there are colonies remaining.

Freezing primary cells

- During early passages cells should be frozen in 1ml aliquots of cells suspended in chilled Recovery™ Cell Culture Freezing Medium.
- Place freezing ampoule upright in freezing box with isopropyl alcohol (Mr. Frosty™) and leave in -70°C for 72 hours. Cells can then be transferred into liquid nitrogen storage.

Resurrecting cell lines from Liquid Nitrogen

- Add 5ml of suitable medium to T25 flask
- Quickly thaw cryovial in 37° water bath
- Transfer to cells into 20ml of medium and spin at 1200rpm for 5mins
- Discard medium and resuspend pellet in 2ml medium
- Transfer to T25 flask

Appendix B. Tissue Microarray Information

1. Clinicopathological data for gynaecological samples in TMA

shorthand	Tumour HPV status	Age at diagnosis	Pathology	Grade	Stage	Sample primary disease or recurrence	Primary Treatment	Outcome of Primary T	Progression/Recurrence
V1	pos	46	Squamous Cell Carcinoma		1B				
C1	pos	49	Adenocarcinoma		1B1				
C2	pos	41	Squamous Cell Carcinoma		1B1				
V2	neg	65	Squamous Cell Carcinoma	1	Recurrence	Recurrence	Mulitple surgeries 1998-2010. Anterior resection 2008. Radical F	n/a	yes
C3	pos	75	Squamous Cell Carcinoma	2	1B2	Primary	vagial hysterectomy with adjuvant CRT	CR	no
V3	neg	74	Squamous Cell Carcinoma within differentia		IB	Primary	surgery	CR	no
C4	pos	53	Squamous Cell Carcinoma	2	IIB	Primary	Chemoradiotherapy	CR	no
V4	neg	75	Squamous Cell Carcinoma	1	Recurrence	Recurrence	Surgery 2009, recurrence 2015 - surgery and adj RT. Furth	n/a	yes
V5	neg	65	Squamous Cell Carcinoma	1	Recurrence	Recurrence	WLE 2013, radical vulvectomy 2015 with adj RT vulva, recc disea	n/a	yes, local 15, pelvic LNs 2016
V6	neg	72	Squamous Cell Carcinoma	2	1B1	Primary	Surgery x 2 & Adjuvant Radiotherapy	uk	yes, lung mets
V7	neg	49	Squamous Cell Carcinoma	3	1B				
C5	pos	39	Squamous Cell Carcinoma	2	1B1				
V8	neg	74	Squamous Cell Carcinoma	2	1B	Primary	surgery	uk	yes
V9	pos	59	Squamous Cell Carcinoma	2	IIIC	Primary	Vulvectomy, RT	CR	no
V10	neg	75	Squamous Cell Carcinoma		I	Primary	Anterior Hemivulvectomy	CR	no
V11	neg	69	Squamous Cell Carcinoma with li	1	1B1				
C6	pos	24	Adenocarcinoma		1B1	Primary	surgery	CR	no
C7	pos	28	Adenocarcinoma		IIA1	Primary	surgery	CR	no
V12	neg	75	Squamous Cell Carcinoma	2	IIIA	Primary	Anterior Hemi-Vulvectomy, then second surgery then adjuvant R	CR	no
V13	neg	69	Squamous Cell Carcinoma		1B	Primary	Hemi-Vulvectomy	CR	no
C8	neg	60	Squamous Cell Carcinoma		1B1	Primary	Surgery	CR	yes
C9	neg	49	Squamous Cell Carcinoma	2	IIIB	Primary	RT	CR/PR Jan 16	yes
C10	neg	57	Adenocarcinoma	1	1B1	Primary	Surgery	CR	no
V14	neg	45	Squamous Cell Carcinoma	2	IIIA	Primary	anterior hemivulvectomy with adjuvant RT	CR	no
C11	pos	29	Squamous Cell Carcinoma	2	IIB	Primary	Surgery and adjuvant chemo RT	CR	no
V15	neg	84	Squamous Cell Carcinoma		Recurrence	Recurrence	numerous surgery 2002 to 2016	n/a	yes
V16	neg	54	Squamous Cell Carcinoma with V	3	1B	Primary	Surgery with adjuvant radiotherapy	CR	no
V17	neg	56	Squamous Cell Carcinoma	1	1B	Primary	Surgery	CR	yes - groin
V18	neg	88	Squamous Cell Carcinoma	1	1B	Primary	Vulvectomy	CR	no
V19	neg	73	Squamous Cell Carcinoma		1A		Posterior Vulvectomy		
V20	neg	75	Squamous Cell Carcinoma		1B	Primary	Surgery 19/12/2016	CR	yes
C12	neg	38	Adenosquamous Carcinoma	2	1B2	Primary	Chemoradiation	CR	no

NC1	neg								
NC2	x								
NC3	neg								
NC4	neg								
NC5	neg								
NC6	neg								
NC7	neg								
NC8	neg								
NC9	neg								
NC10	neg								
NC11	neg								
NC12	neg								
NC13	neg								
NC14	neg								
NC15	neg								
NC16	neg								
NC17	neg								
NC18	x								
NC19	neg								
NC20	neg								
NC21	neg								
NC22	neg								
NC23	neg								
NC24	neg								
NC25	neg								
SKIN	neg								
UTERUS	neg								
OVARY	neg								
STOMACH	neg								
TONGUE	neg								
COLON	neg								
5002	pos	77	Squamous Cell Carcinoma	3	1b1	Primary	surgery	CR	no
5005	pos	29	Squamous Cell Carcinoma	3	1b1	Primary	surgery	CR	no
5007	neg	74	Squamous Cell Carcinoma	2	IIIA	Primary	surgical debulking and radical RT	PD	lung, peritoneal, nodal, bone mets
5008	neg	80	Squamous Cell Carcinoma	1	local recurrence	Recurrence	surgery	n/a	groin recurrence 2017, lung and bone mets 2017
5009	neg	73	Squamous Cell Carcinoma	2	II	Primary	surgery	CR	?declined RT - lost follow up
5010	x	53	Squamous Cell Carcinoma	2	IIIB	Primary	ChemoRT	PR	lung and nodal mets
5015	pos	36	Squamous Cell Carcinoma	3	1B2	Primary	surgery	CR	no
5016	neg	69	Squamous Cell Carcinoma	3	IIIB2	Primary	surgery with nodal dissection followed by RT to groins	CR	no
5018	pos	74	Squamous Cell Carcinoma	1	IB	Primary	ChemoRT	CR	no
5019	pos	36	Squamous Cell Carcinoma	2	IIB	Primary	emergency RT, chemo, completion RT and brachy	CR	yes local and paraaortics
5020	pos	49	Adenocarcinoma	2	IB2	Primary	ChemoRT	CR	no
5021	x	48	Squamous Cell Carcinoma	1	II	Primary	ChemoRT	CR	no
5022	neg	67	Squamous Cell Carcinoma	3	IIIC	Primary	1 x neoadj chemo followed by ChemoRT	n/a	died shortly after completing rx
5023	pos	43	Adenocarcinoma	2	IB1	Primary	surgery	CR	no
5024	neg	82	Squamous Cell Carcinoma	2	recurrence	Recurrence	surgery - previous RT - N.B. think we have sampled tissue from 2	CR	yes -local, required right hemivulvectomy
5026	neg	76	Squamous Cell Carcinoma	2	IIIB	Primary	ChemoRT	PR	yes - local and bone nets
control1									
control2									
control3									
control4									

2. TMA Maps for gynaecological tissue

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
A	V9T	C12T							NC24	NC25	NC23	NC22	NC21								
B	NC8	NC9	NC10	NC11	NC12	NC13	NC14	NC15	NC16	NC17	NC18	NC19	NC20		V1T	V1N	V1D	C1T	C1N	C1D	
C	V1T	V1N	V1D	C1T	C1N	C1D	C2T	C2N	C2D	V2T	V2N	V2D	C3T		NC8	NC9	NC10	NC11	NC12	NC13	NC14
D	V5N	V5T	V4D	V4T	V4N	C4D	C4N	C4T	V3D	V3N	V3T	C3D	C3N		NC21	NC20	NC19	NC18	NC17	NC16	NC15
E	V5D	V6T	V6N	V7T	V7N	C5T	C5N	V8T	V8N	V9T	V9N	V1OT	V10N		NC22	NC23	NC24	NC25			
F	C9T	C8N	C8T	V13N	V13T	V12N	V12T	C7N	C7T	C6N	C6T	V11N	V11T		V1D	V1N	V1T	C12T			
G	NC6	C10T	C10N	V14T	V14N	C11T	C11N	V15T	V15N	V16T	V16N	V17T	V17N		C1T	C1N	C1D	C2T	C2N	C2D	V2T
H	NC7	NC6	NC5	NC4	NC3	NC2	NC1	V20N	V20T	V19N	V19T	V18N	V18T		V3N	V3T	C3D	C3N	C3T	V2D	V2N
I																					
J	NC5		NC7	NC8	NC8	NC10	NC11	NC12	NC13	NC14	NC15	NC16	NC17		V3D	C4T	C4N	C4D	V4T	V4N	V4D
K						NC25	NC24	NC23	NC22	NC21	NC20	NC19	NC18		V7N	V7T	V6N	V6T	V5D	V5N	V5T
L		C12T	V1T	V1N	V1D	C1T	C1N	C1D	C2T	C2N	C2D	V2T	V2N		C5T	C5N	V8T	V8N	C3T	C3N	V10T
M	V4D	V4N	V4T	C4D	C4N	C4T	V3N	V3D	V3T	C3D	C3N	C3T	V2D		C7N	C7T	C6N	C6T	V11N	V11T	V10N
N	V5T	V5N	V5D	V6T	V6N	V7T	V7N	C5T	C5N	V8T	V8N	V9T	V9N		V12T	V12N	V13T	V13N	C8T	C8N	C9T
O	C8T	V13N	V13T	V12N	V12T	C7N	C7T	C6N	C6T	V11N	V11T	V10N	V10T		C11N	C11T	V14N	V14T	C10N	C10T	V9N
P	C8N	C9T	V20N	C10T	C10N	V14T	V14N	C11T	C11N	V15T	V15N	V16T	V16N		V15T	V15N	V16T	V16N	V17T	V17N	V18T
Q	NC7	NC6	NC5	NC4	NC3	NC2	NC1	V20T	V19T	V18N	V18T	V17N	V17T		NC4	NC3	NC2	NC1	V20T	V19T	V18N

	BLANK
	CONTROL
	CASES

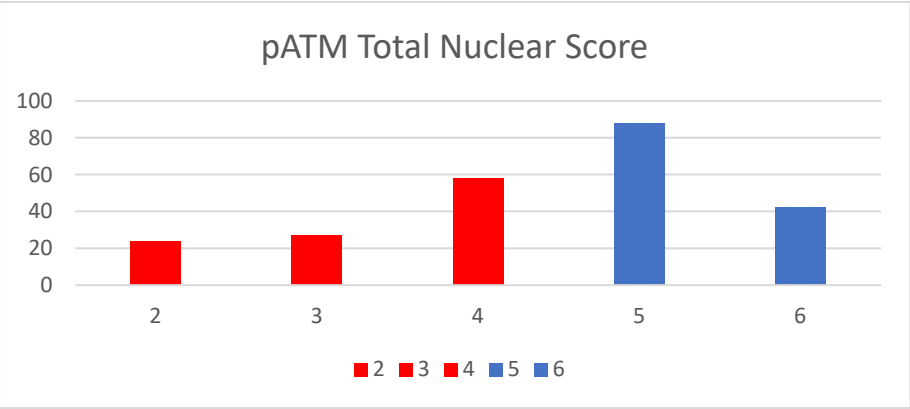
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
0 mm	A					5002T	5002N	5005T		5021N	5022T	5023T	5023N	5024T	5024N	5026T	C2T
1 mm	B					5007N	5007T	5005N		V16T	V8N	V8T	C5T	C4T	V3N	V3T	C2N
2 mm	C			NC12	5025N	5008T	5008N	5009T		V5T	V7T	V7N	NC2	NC5	NC8	5025N	NC13
3 mm	D	control1	control2	control3	control4	5010N	5010T	5009N		NC22	NC21	NC20	control4	control3	control2	control1	NC15
4 mm	E	5025T	5019T	5020T	5021T	5021N	5022T	5023T		5002T	5002N	5005T	5005N	5007T	5007N	5008T	5008N
5 mm	F	V3T	C2N	C2T	5026T	5024N	5024T	5023N		5019T	5025T	5015N	5015T	5010N	5010T	5009N	5009T
6 mm	G	V3N	C4T	C5T	V8T	V8N	V16T	V5T		5020T	5023T	5016T	5016N	5021T	5021N	5022T	5024T
7 mm	H	NC13	NC12	NC8	NC5	NC2	V7N	V7T		C5T	C4T	V3N	V3T	C2N	C2T	5026T	5024N
8 mm	I	NC15	NC16	NC17	NC18	NC19	NC20	NC21		V8T	V8N	V16T	V5T	V7T	V7N	NC2	NC5
9 mm	J	5007T	5007N	5005N	5005T	5002N	5002T	NC22		NC19	NC18	NC17	NC16	NC15	NC13	NC12	NC8
10 mm	K	5008N	5008T	5009T	5009N	5010T	5010N	5015T		NC20	NC21	NC22	5023N	control1	control2	control3	control4
11 mm	L	5021T	5020T	5019T	5025T	5016N	5016T	5015N	5025N	5016N	5016T	5015N	5015T	NC19	NC18	NC17	NC16

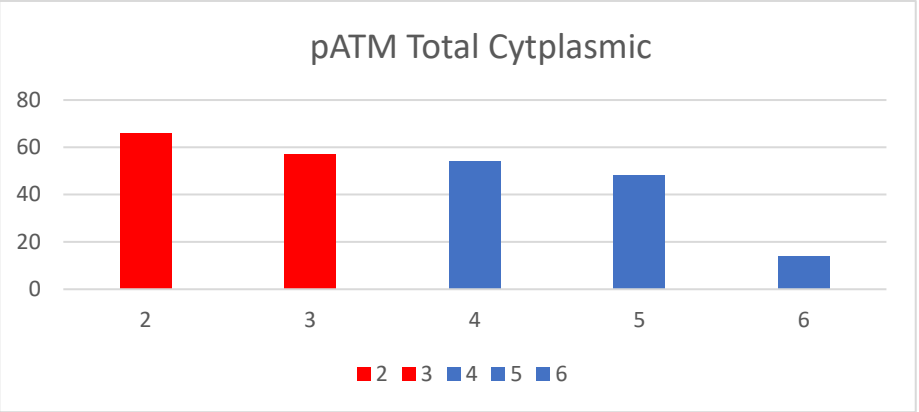
BLANK

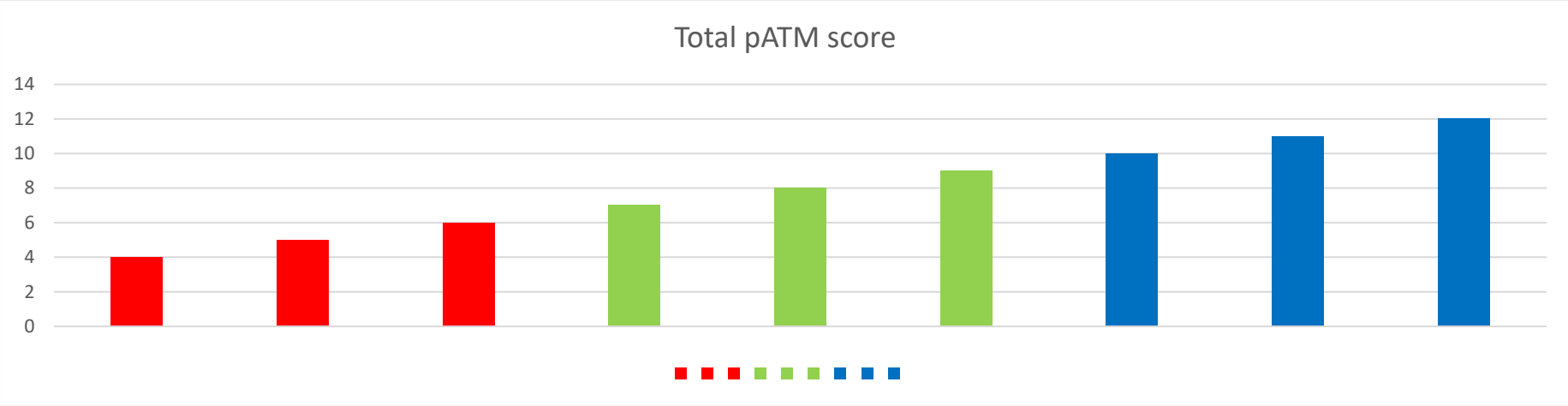
CASES

3. **Spread of data in pATM scoring demonstrating categories chosen for simplified score**

Red = low Green = Medium Blue = high

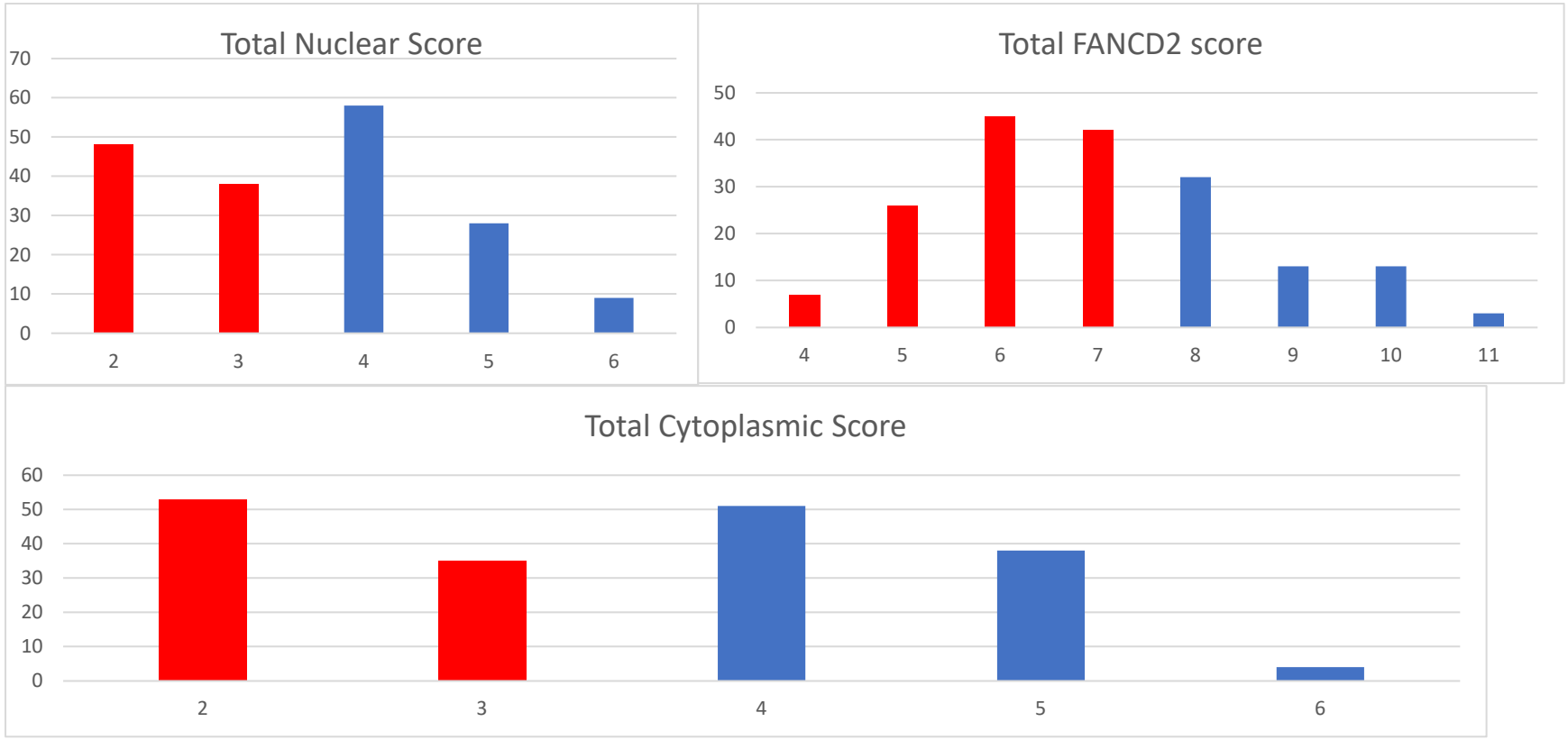






4. Spread of data in FANCD2 scoring demonstrating categories chosen for simplified score

Red = low Blue = high

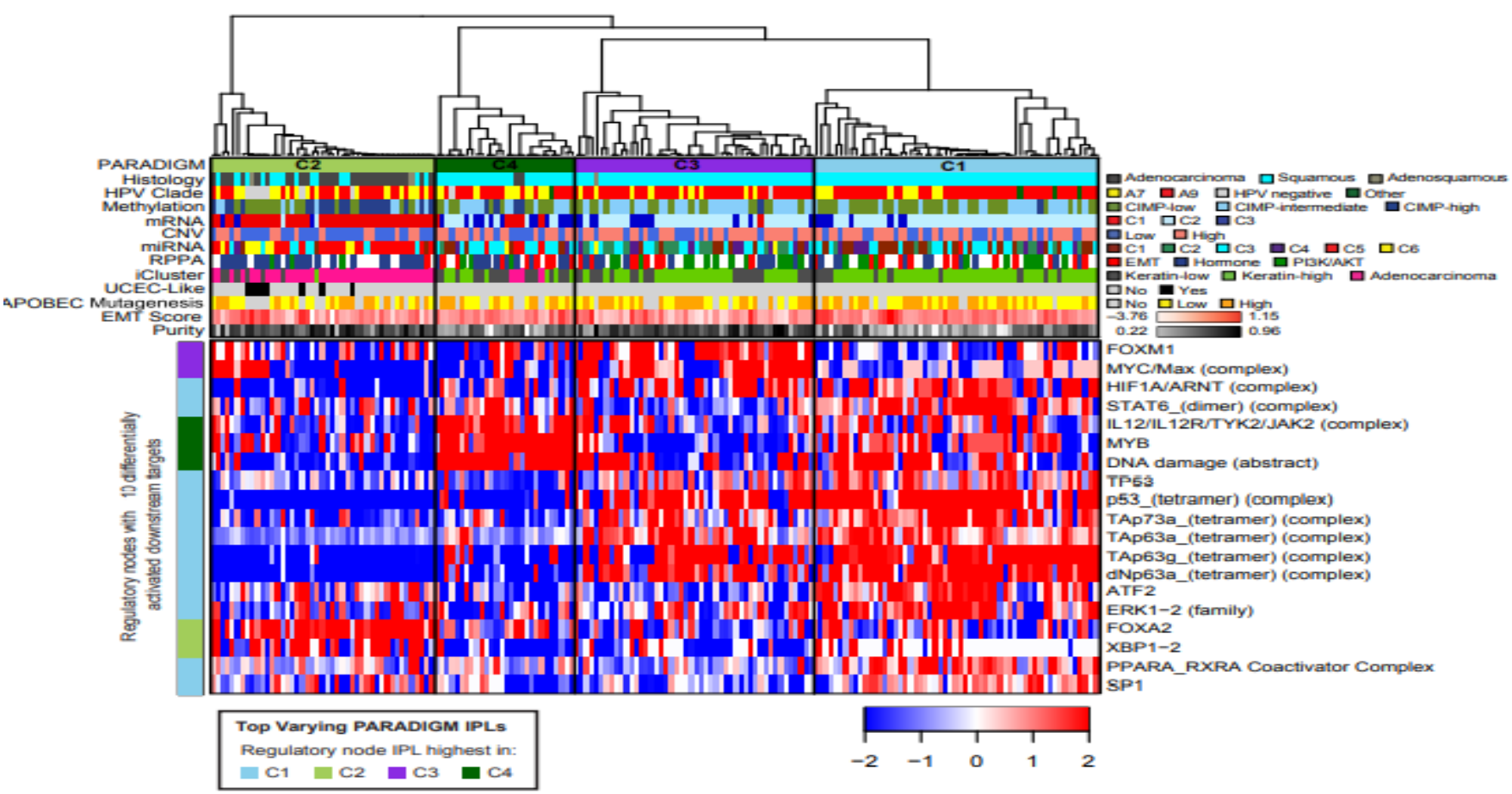


Appendix C. Nanostring PanCancer codeset gene list and supplementary information

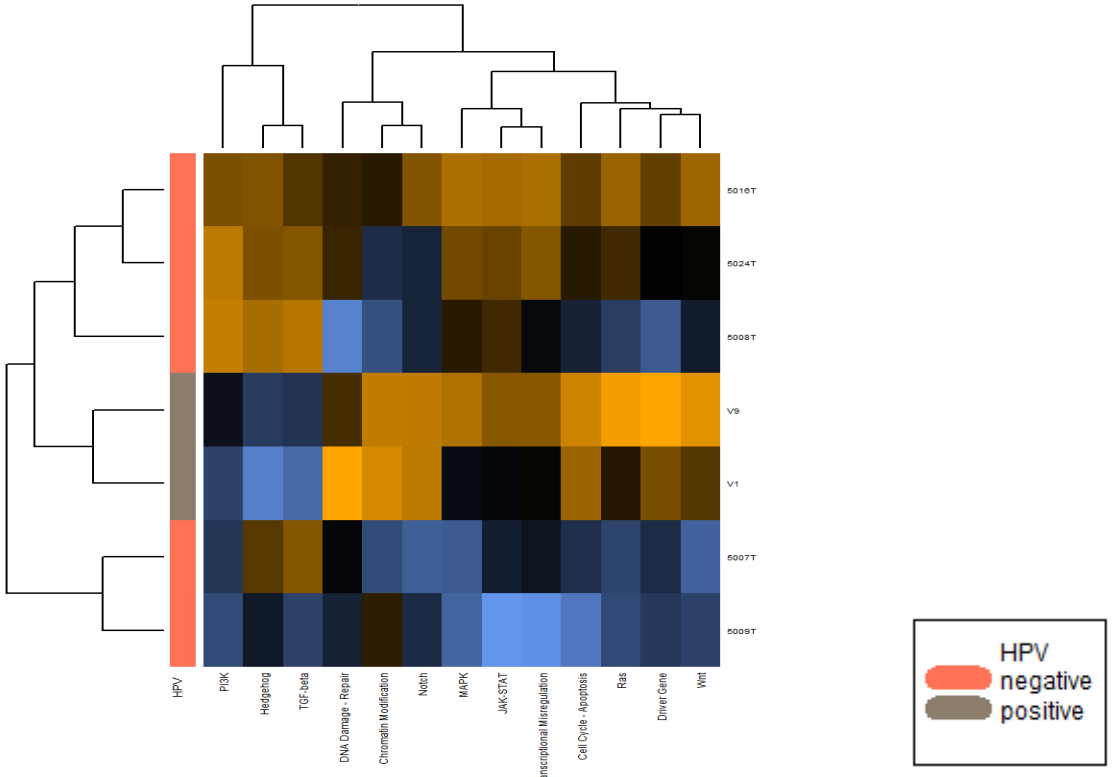
1. Nanostring PanCancer codeset gene list

ABL1	BMP2	CBL	CIC	DLL1	FANCE	FOS	H2AFX	IFNA7	INHBB	LEFTY2	MFNG	NPM2	PKMYT1	PRKACG	RFC4	SOCS2	TMPRSS2	WNT6
ACVR1B	BMP4	CBLC	CLCF1	DLL3	FANCF	FOSL1	H3F3A	IFNG	IRAK2	LEP	MGMT	NR4A1	PLA1A	PRKAR1B	RHOA	SOCS3	TNC	WNT7A
ACVR1C	BMP5	CCNA1	CNTFR	DLL4	FANCG	FOXL2	H3F3C	IGF1	IRAK3	LEPR	MLF1	NR4A3	PLA2G10	PRKAR2A	RIN1	SOS1	TNF	WNT7B
ACVR2A	BMP6	CCNA2	COL11A1	DNMT1	FANCL	FOXO4	HDAC1	IGF1R	IRS1	LFNG	MLH1	NRAS	PLA2G2A	PRKAR2B	RNF43	SOS2	TNFAIP3	WT1
AKT1	BMP7	CCNB1	COL11A2	DNMT3A	FAS	FST	HDAC10	IGFBP3	ITGA2	LIF	MLLT3	NSD1	PLA2G3	PRKCA	RPA3	SOST	TNFRSF10A	XPA
AKT2	BMP8A	CCNB3	COL1A1	DTX1	FASLG	FUBP1	HDAC11	IKBKB	ITGA3	LIFR	MLLT4	NTF3	PLA2G4A	PRKCB	RPS27A	SOX17	TNFRSF10B	XRCC4
AKT3	BMPR1B	CCND1	COL1A2	DTX3	FBXW7	FUT8	HDAC2	IKBKG	ITGA6	LIG4	MMP3	NTHL1	PLA2G4C	PRKCG	RPS6KA5	SOX9	TNFRSF10C	ZAK
ALK	BNIP3	CCND2	COL24A1	DTX4	FEN1	FZD10	HDAC4	IL10	ITGA7	LRP2	MMP7	NTRK1	PLA2G4E	PRKDC	RPS6KA6	SP1	TNFRSF10D	ZBTB16
ALKBH2	BRAF	CCND3	COL27A1	DUSP10	FGF1	FZD2	HDAC5	IL11	ITGA8	LTBP1	MMP9	NTRK2	PLA2G4F	PRKX	RRAS2	SPOP	TNFSF10	ZBTB32
ALKBH3	BRCA1	CCNE1	COL2A1	DUSP2	FGF10	FZD3	HDAC6	IL11RA	ITGA9	MAD2L2	MNAT1	NUMBL	PLA2G5	PRL	RUNX1	SPP1	TNN	ZIC2
AMER1	BRCA2	CCNE2	COL3A1	DUSP4	FGF11	FZD7	HELLS	IL12A	ITGB3	MAML2	MPL	NUPR1	PLAT	PRLR	RUNX1T1	SPRY1	TNR	
AMH	BRIP1	CCNO	COL4A3	DUSP5	FGF12	FZD8	HES1	IL12B	ITGB4	MAP2K1	MPO	OSM	PLAU	PRMT8	RXRG	SPRY2	TP53	
ANGPT1	C19orf40	CCR7	COL4A4	DUSP6	FGF13	FZD9	HES5	IL12RB2	ITGB6	MAP2K2	MSH2	PAK3	PLCB1	PROM1	SETBP1	SPRY4	TPO	
APC	CACNA1C	CD14	COL4A5	DUSP8	FGF14	GADD45A	HGF	IL13	ITGB7	MAP2K4	MSH6	PAK7	PLCB4	PTCH1	SETD2	SRSF2	TRAF7	
APH1B	CACNA1D	CD19	COL4A6	E2F1	FGF16	GADD45B	HHEX	IL13RA2	ITGB8	MAP2K6	MTOR	PAX3	PLCE1	PTCRA	SF3B1	SSX1	TSC1	
AR	CACNA1E	CD40	COL5A1	E2F5	FGF17	GADD45G	HHIP	IL15	JAG1	MAP3K1	MUTYH	PAX5	PLCG2	PTEN	SFN	STAG2	TSHR	
ARID1A	CACNA1G	CDC14A	COL5A2	EFNA1	FGF18	GAS1	HIST1H3B	IL19	JAG2	MAP3K12	MYB	PAX8	PLD1	PTPN11	SFRP1	STAT1	TSLP	
ARID1B	CACNA1H	CDC14B	COL6A6	EFNA2	FGF19	GATA1	HIST1H3G	IL1A	JAK1	MAP3K13	MYC	PBRM1	PML	PTPN5	SFRP2	STAT3	TSPAN7	
ARID2	CACNA2D1	CDC25A	COMP	EFNA3	FGF2	GATA2	HIST1H3H	IL1B	JAK2	MAP3K14	MYCN	PBX1	POLB	PTPRR	SFRP4	STAT4	TTK	
ARNT2	CACNA2D2	CDC25B	CREB3L1	EFNA5	FGF20	GATA3	HMGA1	IL1R1	JAK3	MAP3K5	MYD88	PBX3	POLD1	PTTG2	SGK2	STK11	U2AF1	
ASXL1	CACNA2D3	CDC25C	CREB3L3	EGF	FGF21	GDF6	HMGA2	IL1R2	JUN	MAP3K8	NASP	PCK1	POLD4	RAC1	SHC1	STMN1	UBB	
ATM	CACNA2D4	CDC6	CREB3L4	EGFR	FGF22	GHR	HNF1A	IL1RAP	KAT2B	MAPK1	NBN	PCNA	POLE2	RAC2	SHC2	SUV39H2	UBE2T	
ATR	CACNB2	CDC7	CREB5	EIF4EBP1	FGF23	GLI1	HOXA10	IL20RA	KDM5C	MAPK10	NCOR1	PDGFA	POLR2D	RAC3	SHC3	SYK	UTY	
ATRX	CACNB3	CDH1	CREBBP	ENDOG	FGF3	GLI3	HOXA11	IL20RB	KDM6A	MAPK12	NF1	PDGFB	POLR2H	RAD21	SHC4	TBL1XR1	VEGFA	
AXIN1	CACNB4	CDK2	CRLF2	EP300	FGF4	GNA11	HOXA9	IL22RA1	KIT	MAPK3	NF2	PDGFC	POLR2J	RAD50	SIN3A	TCF3	VEGFC	
AXIN2	CACNG1	CDK4	CSF1R	EPHA2	FGF5	GNAQ	HPGD	IL22RA2	KITLG	MAPK8	NFATC1	PDGFD	PPARG	RAD51	SIRT4	TCF7L1	VHL	
B2M	CACNG4	CDK6	CSF2	EPO	FGF6	GNAS	HRAS	IL23A	KLF4	MAPK8IP1	NFE2L2	PDGFRA	PPARGC1A	RAD52	SIX1	TCL1B	WEE1	
BAD	CACNG6	CDKN1A	CSF3	EPOR	FGF7	GNG12	HSP90B1	IL23R	KMT2C	MAPK8IP2	NFKB1	PDGFRB	PPP2CB	RAF1	SKP1	TET2	WHSC1	
BAIAP3	CALML3	CDKN1B	CSF3R	ERBB2	FGF8	GNG4	HSPA1A	IL24	KMT2D	MAPK9	NFKBIA	PGF	PPP2R1A	RASA4	SKP2	TFDP1	WHSC1L1	
BAMBI	CALML5	CDKN1C	CTNNB1	ERCC2	FGF9	GNG7	HSPA2	IL2RA	KRAS	MAPT	NFKBIZ	PHF6	PPP2R2B	RASAL1	SMAD2	TGFB1	WIF1	
BAP1	CALML6	CDKN2A	CUL1	ERCC6	FGFR1	GNGT1	HSPA6	IL2RB	LAMA1	MCM2	NGF	PIK3CA	PPP2R2C	RASGRF1	SMAD3	TGFB2	WNT10A	
BAX	CAMK2B	CDKN2B	CXXC4	ETS2	FGFR2	GPC4	HSPB1	IL3	LAMA3	MCM4	NGFR	PIK3CB	PPP3CA	RASGRF2	SMAD4	TGFB3	WNT10B	
BCL2	CAPN2	CDKN2C	CYLD	ETV1	FGFR3	GRB2	IBSP	IL3RA	LAMA5	MCM5	NKD1	PIK3CD	PPP3CB	RASGRP1	SMAD9	TGFBR2	WNT11	
BCL2A1	CARD11	CDKN2D	DAXX	ETV4	FGFR4	GRIA3	ID1	IL5RA	LAMB3	MCM7	NODAL	PIK3CG	PPP3CC	RASGRP2	SMARCA4	THBS1	WNT16	
BCL2L1	CASP10	CEBPA	DDB2	ETV7	FIGF	GRIN1	ID2	IL6	LAMB4	MDC1	NOG	PIK3R1	PPP3R1	RB1	SMARCB1	THBS4	WNT2	
BCOR	CASP12	CEBPE	DDIT3	EYA1	FLNA	GRIN2A	ID4	IL6R	LAMC2	MDM2	NOS3	PIK3R2	PPP3R2	RBX1	SMC1A	THEM4	WNT2B	
BDNF	CASP3	CHAD	DDIT4	EZH2	FLNC	GRIN2B	IDH1	IL7	LAMC3	MECOM	NOTCH1	PIK3R3	PRDM1	RELA	SMC1B	TIAM1	WNT3	
BID	CASP7	CHEK1	DKK1	FANCA	FLT1	GSK3B	IDH2	IL7R	LAT	MED12	NOTCH2	PIK3R5	PRKAA2	RELN	SMC3	TLR2	WNT4	
BIRC3	CASP8	CHEK2	DKK2	FANCB	FLT3	GTF2H3	IFNA17	IL8	LEF1	MEN1	NOTCH3	PIM1	PRKACA	RET	SMO	TLR4	WNT5A	
BIRC7	CASP9	CHUK	DKK4	FANCC	FN1	GZMB	IFNA2	INHBA	LEFTY1	MET	NPM1	PITX2	PRKACB	RFC3	SOCS1	TLX1	WNT5B	

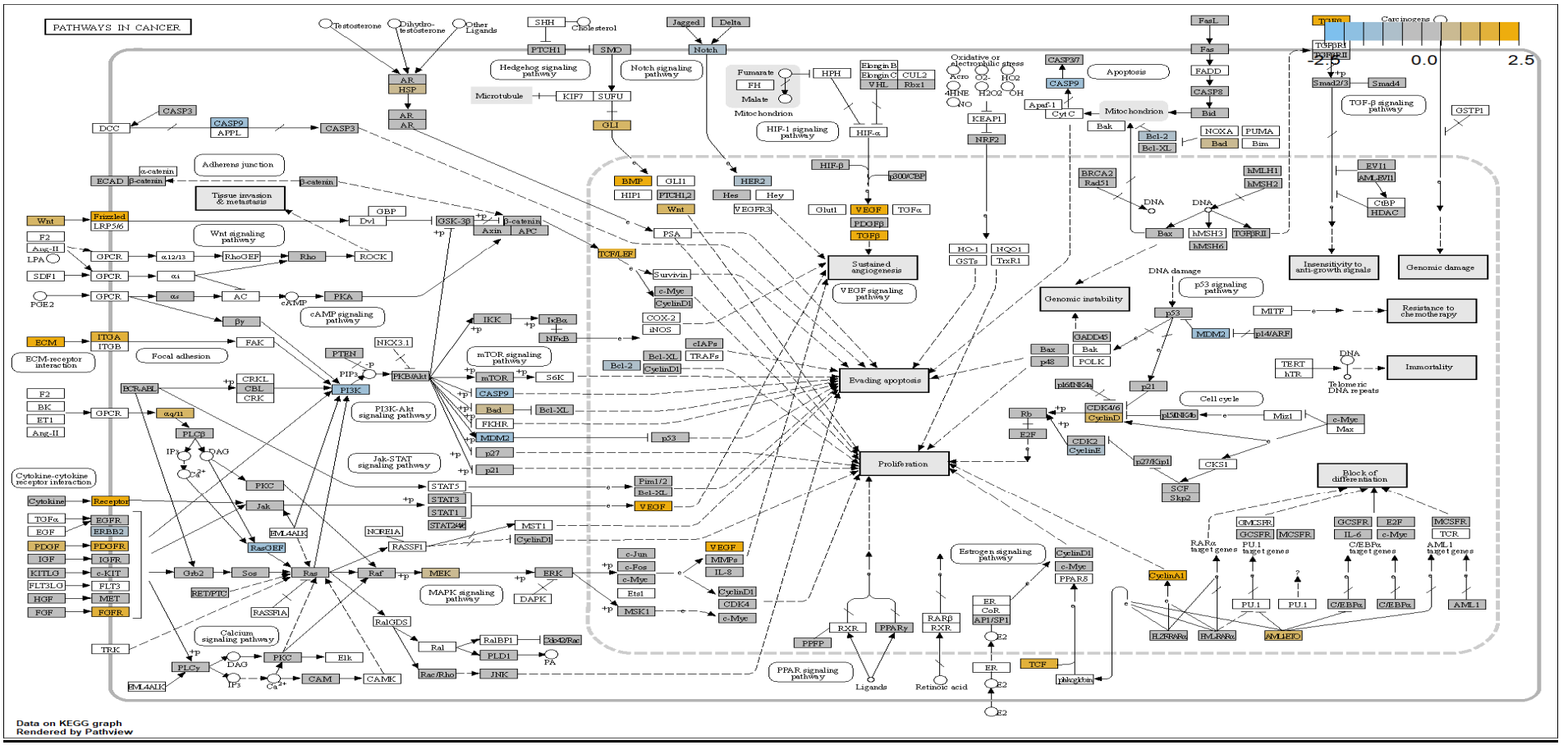
2. TCGA Molecular profiling of cervical cancers



3. Heatmap of mean pathway scores of vulval cancers denoted by HPV status



4. Differential expression of Group A tumours versus Group B overlaid on cancer pathway map



Appendix D. COMICE protocol in progress publication submission and COMICE Protocol in progress publication submission

1. COMICE protocol in progress publication submission

COMICE: A study protocol for a randomised double blind placebo controlled phase II clinical trial of cediranib and olaparib maintenance therapy in advanced or recurrent cervical cancer

Authors

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Affiliations

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Clatterbridge Cancer Centre, Liverpool, UK

Abstract:

Introduction: Patients with advanced or recurrent cervical cancer have limited therapeutic options following treatment with platinum-based chemotherapy. Anti-angiogenic therapies have shown promise in cervical cancer, and these agents are hypothesised to have a synergistic effect when used in combination with PARP inhibitors, in tumours with underlying DNA repair deficiencies. Almost all cervical cancers are associated with Human Papillomavirus which alters cellular DNA repair pathways which may in turn result in sensitivity to PARP inhibitor therapy. We hypothesise that a combination of cediranib and olaparib maintenance therapy following chemotherapy for advanced and recurrent cervical cancer could improve outcomes for this patient group.

Methods and analysis: COMICE is a Phase II multicentre, randomised, placebo controlled, double blind clinical trial. The study will take place in approximately 23 UK oncology centres and 108 patients will be randomised in a 1:1 fashion to the experimental arm of cediranib and olaparib tablets, or matched placebo. The primary outcome measure for the study is progression-free survival with secondary outcome measures being overall survival, best objective response and quality of life. Safety outcomes will also be collected including the occurrence of Grade 3+ Toxicity, occurrence of Serious Adverse Events and withdrawal due to toxicity.

Ethics and Dissemination: This protocol was approved by the North West - Greater Manchester South Research Ethics Committee and the Health Research Authority.

At study completion the results will be disseminated through publication in peer-reviewed journals and at scientific conferences.

Trial Registration; ISRCTN No.10841582

Strengths and limitations of this study

- The COMICE trial is a much needed opportunity to study targeted agents in patients with advanced cervical cancer who have limited therapeutic options beyond first line therapy.

- The study benefits from having a translational research component to enable the molecular analysis of tumour and plasma samples with the aim of identifying biological characteristics of the disease and predictive biomarkers for study drug response.

- Due to limitations in sample size in this phase II study, it is not possible to evaluate the treatment drugs as monotherapy in addition to in combination.

- Trial recruitment and conduct has been impacted by the COVID 19 pandemic, but recruitment has now resumed following protocol amendments to ensure the safety of trial participants.

Introduction

Cervical cancer is the 4th most common female cancer worldwide and remains a significant global health burden (1). Early stage and locally advanced tumours may be cured with radical surgery, chemoradiotherapy or both, but unfortunately there are limited options beyond platinum-based chemotherapy for patients with metastatic disease or persistent/recurrent disease (2). The benefits from such chemotherapy are modest with a response rate (RR) of 30-35%, progression free survival of 5.8 months and a median overall survival of less than a year (3). Second line chemotherapy has to date shown poor efficacy with no improvement in survival demonstrated, clinical trials with targeted agents are urgently needed to improve the outlook for this group of patients.

Angiogenesis is a key hallmark in cervical tumour progression involving the formation of neo-vasculature to facilitate tumour growth. Pro-angiogenic signalling is upregulated in cervical cancers and high levels of tumour angiogenic factors are an adverse prognostic factor and associated with poor survival (4-6). The phase III randomised controlled trial GOG 240 demonstrated a 3.7 month improvement in median overall survival (OS) with the addition bevacizumab, a monoclonal antibody targeting vascular endothelial growth factor (VEGF), to combination chemotherapy (7). The ORR and progression free survival (PFS) were also improved in patients receiving bevacizumab compared to those receiving chemotherapy alone. This led to the approval of bevacizumab for up to 6 cycles in combination with chemotherapy for metastatic cervical cancer.

Small molecule inhibition of the tyrosine kinase activity of VEGF receptors has also shown promise in cervical cancer with cediranib, a highly potent inhibitor of the tyrosine kinase activity of VEGF receptors 1-3 (VEGFR 1-3), demonstrating an

increased PFS and RR when added to combination chemotherapy in advanced cervical cancer in the CIRCCa study (8). Following completion of combination chemotherapy, cediranib was continued as a maintenance phase until disease progression. The median PFS was 35 weeks and 29 weeks in the cediranib and placebo arms respectively (HR 0.58 p= 0.032). In the maintenance phase cediranib showed no detriment to quality of life (QOL) and manageable increased toxicity (specifically diarrhoea and hypertension). An alternative VEGFR2 tyrosine kinase inhibitor, apatinib, has also been evaluated in a small, single arm phase II study in patients who failed to respond first line therapy, and demonstrates some activity in this setting with an objective response rate of 15% and a disease control rate of 35% (9).

In both the GOG 240 and CIRCCa study the anti-angiogenic agents were continued until progression either alongside chemotherapy (GOG240) or as a monotherapy (CIRCCa). There is no approved maintenance treatment beyond 6 cycles of chemotherapy in the UK, as there are no available OS data to support the use of either bevacizumab or cediranib monotherapy in this setting. We hypothesise that outcomes in this patient group could be improved through the use of an antiangiogenic agent as a maintenance treatment. However the benefits of single antiangiogenic agents are modest and we hypothesise may be improved through rational combination with a Poly (ADP-ribose) polymerase inhibitor (PARPi).

PARP inhibitors have potential activity in cancers deficient in DNA repair genes or signalling pathways that mediate DNA repair. Synthetic lethality can occur with use of PARP inhibitors in the context of underlying deficiency in double strand DNA repair, as the loss of PARP activity abrogates the ability of cells to maintain genomic integrity in the face of recurrent double strand DNA (dsDNA) breaks. PARP inhibitors demonstrate a selectivity for cells harbouring homologous recombination (HR) deficiencies such as those with BRCA mutations, although synthetic lethality may be more broadly applied to cancers with an impaired HR pathway for other reasons, such as disruption of the Fanconi Anaemia pathway (10). There is an emerging body of evidence to suggest that Human Papillomavirus (HPV), the primary aetiological agent in almost all cervical cancers, can cause disruption of dsDNA repair and homologous recombination. Current therapeutic modalities for cervical cancers exploit the sensitivity of these tumours to DNA damaging agents such as ionising radiation and platinum chemotherapeutic agents.

Studies suggest that the presence of high-risk HPV oncoproteins (E6/E7) can cause dysregulation of DNA repair checkpoints, resulting in HR protein activation outside of the S/G2 phase of the cell cycle where it cannot be performed successfully (11). Cells expressing E6/E7 can also attenuate the cellular response to DNA damage by impairing the localisation of FANCD2 to sites of cellular DNA damage, consequently preventing foci formation of homologous recombination proteins such as RAD51 and PALB2 (12). We therefore postulate that the action of HPV oncoproteins render HPV associated cancers susceptible to PARP inhibition.

Preclinical evidence suggests that PARP inhibitors and anti-angiogenic agents may act synergistically, as cediranib has been shown to down-regulate HR proteins in mouse xenografts (13). In addition, the combination of olaparib and cediranib has previously been evaluated in recurrent platinum sensitive ovarian cancer in a phase II clinical study, which demonstrated that these agents can be delivered safely in

combination and with encouraging efficacy signal (14, 15). We therefore hypothesize that this combination may result in improved control of disease in patients with recurrent/metastatic cervical cancer whose disease has responded or remained stable after treatment with standard platinum based chemotherapy.

Methods and Analysis

Study Design

This is a Phase II multicentre, randomised, parallel group, placebo controlled, double blind, clinical trial. The study will be carried out in approximately 23 UK oncology centres with competitive recruitment. 108 patients will be randomised in a 1:1 fashion. A flow diagram of the trial schema is shown in Figure 1. There are no approved maintenance therapies for cervical cancer in the UK and therefore best supportive care is the comparator until disease progression.

Patient and Public Involvement

During study development review of trial design, trial protocol and patient information sheet occurred within two separate Patient Advisory Groups, with suggested amendments being considered and appropriate changes made. The study also has an appointed patient representative on the trial steering committee and all protocols amendments are reviewed by a minimum of three patient representatives.

Patient Recruitment

For inclusion into this study patients must be (1) over 18 years of age (2) have histologically proven carcinoma of the cervix (squamous, adenocarcinoma or mixed adeno/squamous) (3) an ECOG performance status of 0 or 1 (4) radiological confirmation of either a complete response, partial response or stable disease following completion of first line platinum based chemotherapy +/- bevacizumab for advanced /recurrent disease (as evaluated by RECIST 1.1) performed within 28 days prior to randomisation (5) have adequate haematological and biochemical function as defined in the protocol (Additional material 1) (6) may have received previous chemoradiotherapy and neo-adjuvant chemotherapy given with a curative intent (7) have a life expectancy >12 weeks (8) be able to swallow and retain oral medications and without gastrointestinal (GI) illnesses that would preclude absorption of cediranib or olaparib (9) provide written informed consent.

Study exclusion criteria include: (1) disease that is potentially treatable with exenterative surgery (2) disease relapse confined to the pelvis after radical surgery in circumstance where radiotherapy or chemoradiotherapy would be appropriate

(3) prior treatment with anti-angiogenic agents (with the exception of bevacizumab given as part of first line chemotherapy) (4) persisting ≥Grade 2 CTCAE from previous anti-cancer previous systemic anti-cancer therapy except haematological toxicity and alopecia (5) history of other malignancy within the previous 5 years except for those outlined in the protocol (6) pregnant or lactating women (7) fertile woman of childbearing potential not willing to use adequate contraception (as defined by the protocol) for the study duration and at least six months afterwards (7) evidence of uncontrolled infection (8) history of pelvic fistulae or history of abdominal fistula that has been surgically corrected within 6 months of starting treatment (7) sub-acute or acute intestinal obstruction (8) major surgery within 28 days or anticipated while on study (9) non-healing wound, ulcer or bone fracture (10) active bleeding (11) history or evidence of thrombotic or haemorrhagic disorders including history of stroke or transient ischemic attack within 6 months (12) proteinuria > 1+ on dipstick on two consecutive dipsticks taken no less than 1 week apart, unless urinary protein is <1.5g in a 24 hour period.

Each participating centre must have at least one lead clinician with a specific interest in, and responsibility for supervising, treating and managing patients with advanced/recurrent cervical cancer and ensure that sufficient time, staff and adequate facilities are available for the study including a suitable multidisciplinary team meeting to identify patients. Suitable patients will be referred to the research team for screening and will be given a patient information sheet (PIS) to consult regarding trial participation. Should the patient be eligible and wish to proceed with the study, written informed consent will be taken by an appropriate clinician and the patient randomised. Screening must be completed, and the patient randomised within 8 weeks of the last dose of chemotherapy. Consent will also be sought for collection/use of biological samples for translational research.

The randomisation code list will be generated by the trial statistician with equal allocation to the two treatment arms stratified on previous bevacizumab use prior Chemo-radiotherapy and previous neoadjuvant chemotherapy. Each patient randomly allocated a study treatment will be given a unique study number through an interactive web response system which is managed and maintained by the trials centre.

Interventions

Study participants must start their first trial treatment no later than 14 days after randomisation. Patients will be randomised between the experimental arm of cediranib 20mg OD (5 consecutive days out of 7 days) and Olaparib 300mg BD tablets (continuous) or matched placebo.

Patients will be seen on a 4-weekly basis during the time they are on active treatment and will be treated until disease progression. Treatment will be stopped earlier if the patient withdraws from the study due to toxicity, patient choice,

pregnancy, allergy, or a treatment break of over 3 weeks is necessitated. If a participant consents, EDTA blood samples will be collected at baseline, treatment review visits (cycles 1 and 2) and at disease progression for use in translational research.

Drug administration may be halted, and drug dosage modified in the event of drug related adverse events as deemed appropriate by the Investigator. Olaparib has a two-level dose reduction schedule with the doses altered to 300mg and 200mg daily and a further reduction to 200mg BD if required. Cediranib dosage can be reduced to 15mg daily (5 consecutive days out of 7 days). Guidance on management of specific toxicities related to study drugs, including dose reduction, are outlined within the protocol. Cediranib is required to be halted 2 weeks prior to any invasive procedure where there is a risk of bleeding. To assess the compliance with trial treatment, patients will be asked to return a drug diary and any unused tablets to their clinic visits.

Investigators, site staff and the COMICE trial team (with the exception of a trial centre IT representative, unblinded Coordinators and the independent company providing the trial drugs) will remain blinded with regard to the randomised treatment assignments up to database lock. The trial statistician will be partially blinded to produce reports for trial monitoring. The treatment code must not be broken except in medical emergencies when the appropriate management of the patient necessitates knowledge of the treatment randomisation. Unblinding will be performed by the trial centre's unblinded coordinators or by the sponsor's trial pharmacy outside of office hours.

Outcomes

The primary outcome measure is progression-free survival (PFS), selected due to the nature of the experimental arm (maintenance therapy). Secondary outcome measures are overall survival (OS), best objective response and quality of life. Safety outcomes will also be collected including the occurrence of Grade 3+ Toxicity, occurrence of Serious Adverse Events and withdrawal due to toxicity. The PFS in the control arm is based on the placebo arm in the CIRCCa trial but estimating survival conditional on being progression-free at three months in accordance with the eligibility criteria for COMICE. This corresponds to progression-free survival of 70% at 3 months and 15% at 6 months. Using a log rank test to detect a hazard ratio of 0.6 (corresponding to a change in median survival from 3.77 to 4.64 months) at a 5% one-sided significance with 80% power, assuming equal allocation, 100 events are needed (Stata power log rank command). The recruitment of 108 study participants is expected in practice to yield 102 events giving a power of 82% (Stata artsurv package).

Data Collection and Management

Study specific screening activities will be performed after patients have consented to study participation and signed the informed consent form. These include confirmation of histological diagnosis (centres will also be asked to send formalin fixed paraffin embedded (FFPE) tissue related to the diagnosis of cervical cancer for

translational research with patient consent), demographics and medical history, concomitant medication review and check, concurrent condition review and check, physical examination and medical Review, ECOG Performance Status, 12-lead ECG, CT scan of chest with RECIST, CT or MRI Scan of abdomen and pelvis with RECIST , haematological/clinical biochemistry, urinalysis, vital signs, height and weight, Quality of Life questionnaire and a pregnancy test (women of child-bearing potential only).

Treatment visits occur every 4 weeks with some changes being necessitated by the COVID-19 pandemic. Patients are now advised to attend a medical setting (treating hospital or GP) for their 4-weekly for blood tests, urinalysis, and blood pressure only, with all other assessments performed by the research nurse over the telephone. Telephone consultations will include the Quality of Life questionnaire, ECOG performance status, concomitant medication review, drug compliance review and adverse events review.

Following remote treatment review appointments, arrangements can be made to courier trial drugs to the patients' home if verbal consent is obtained. EDTA samples for research use can only be collected at hospital sites.

The primary outcome of progression free survival will be assessed by a RECIST V1.1 reported CT scan (chest) and CT or MRI of the abdomen (with a consistent modality used throughout study treatment). The window for these scans has been extended from 8 weeks to every 8-12 weeks to make this more achievable in view of the COVID pandemic. Toxicities will be documented using the National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events (CTCAE) Version 4.03 for the reporting of adverse events. Quality of life data will be collected using the FACT-Cx scale, with patients being encouraged to complete questionnaires at appointments to improve compliance.

Trial data will be captured by remote data entry onto the electronic case report form (eCRF). The trial database will be subject to automated validation and central monitoring, and any discrepancies or data amendments will have a complete audit trail. The trial centre will request consent from all patients to obtain information from the NHS Information Centre (Medical Research Information Service) to follow patient progress if this is not available from their hospital or General Practitioner (GP), including patients who withdraw from the study and do not want to proceed to the follow-up phase.

Data Analysis

Statistical analyses will be performed using Stata, SAS or R. The trigger for the final analysis will be 7 months after the last recruited patient's randomisation date unless all patients are off study before that date, or if early termination has been decided. The primary outcome of PFS and secondary outcome of OS will be carried out on an Intention to treat basis using a log rank test stratified on prior therapy. Significance levels for the primary endpoint and overall survival will be 5% one-sided, and both PFS and OS will be presented with 90% two-side confidence intervals by treatment arm. No adjustment will be made for multiple comparisons.

The secondary outcome of best objective response rate will be analysed by a generalised linear model with identity link and binomial family using robust standard error. This analysis will give difference in proportions directly (and NNT by reciprocal transform). Quality of Life outcomes will be measured by the Trial Outcome Index (TOI) defined as the sum of the Physical Well-Being, Functional Well-being and Cervical Cancer-specific subscales. Data will be scored according to the algorithm described in the FACIT scoring instruction, including item non-response. The TOI will be summarised by treatment group over time as area under the curve (AUC) and overall AUC values will be compared using a stratified Mann-Whitney test based on Stouffer's method. Missing values that remain on the Trial Outcome index and missing response data will be replaced by multiple imputation. The number and percentage of patients reporting a Serious Adverse Event (SAE) and Grade 3 or higher toxicity will be separately summarised by treatment arm overall and by preferred term. "Exact" two-sided p-values will be displayed with a False Discovery Rate of 0.05. In addition, the number and percentage of patients discontinuing study treatment or withdrawing from the trial due to SAE or toxicity will be presented by treatment arm, again displayed with "Exact" two-sided p-values. There are no pre-specified subgroup analyses planned.

Monitoring

All Adverse Events (AEs) that occur from randomisation to 30 days following the last administration of study drugs and any ongoing events after treatment will be documented. All SAEs will be reviewed by independent Clinical Co-ordinators to assess causality and expectedness and any SUSARs reported to the sponsor, ethics committees and regulatory authorities. Monthly safety reports will be generated by the trials centre, to allow oversight of adverse events by the Trial Management Group (TMG) and Independent Data and Safety Monitoring Committee (IDSMC) and annual safety reports will be submitted to the MHRA and REC. The ISDMC will provide a recommendation to the

Trial Steering Committee (TSC), who has the authority to make decisions concerning the continuation of the study. Central and trial centre monitoring will be conducted to ensure protection of patients participating in the trial, and that trial procedures including study treatment administration and sample and data collection processes are of high quality and meet sponsor and regulatory requirements as appropriate.

Ethics and dissemination

This study protocol has received ethical approval from the North West – Greater Manchester South Research and Ethics Committee (IRAS 209375). The CI will update the ethics committee of any new information related to the study drug and any protocol modifications that are required. Legal indemnity is provided by the NHS indemnity scheme and Product liability is given by Astra Zeneca as the drug manufacturer. This study is also endorsed by Cancer Research UK.

All results of this study will be analysed together and published as soon as possible. The Trial Management Group will form the basis of the Writing Committee and advise on the nature of publications. All investigators who recruit patients will be named as co-authors, and named authors will include the Chief Investigator, Statisticians and Trial Managers. All investigators will be required to declare financial and other competing interests.

Trial Status

Protocol version 4 19th March 2020. Recruitment started 13/09/2018, expected recruitment completed by 30/11/2022.

List of abbreviations

RR – response rate

OS - overall survival

PFS – progression free survival

VEGF - vascular endothelial growth factor

VEGFR - vascular endothelial growth factor receptor

QOL – quality of life

PARPi- poly (ADP-ribose) polymerase inhibitor

dsDNA - double strand DNA

HR - homologous recombination

HPV - human Papillomavirus

CTCAE – common terminology criteria for adverse events

PIS – patient information sheet

FFPE - formalin fixed paraffin embedded

TOI - trial outcome Index

AUC - area under the curve

SAE - serious adverse event

AE - adverse event

TMG - trial management group

IDSMC - independent data and safety monitoring committee

TSC - trial steering committee

Declarations

Authors' contributions: The study design was conceived by RL and the protocol written by RL, JS, LC, JD, and MG with assistance of the Liverpool Clinical Trials Unit. Manuscript prepared by L Cossar and reviewed by the Authors and the trial Sponsor (Clatterbridge Cancer Centre).

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Competing interest's statement. RL sits on the ICON 9 TMG, advisory boards for GSK and AZ and has received travel reimbursements from GSK and AZ. JS has received research funding from AZ.

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2. COMICE translational research proposal

Proposed COMICE trial protocol submitted to funders (2017)

The COMICE phase II randomised controlled study is a commercial multi-centre UK trial investigating the combination of olaparib and cediranib as maintenance therapy following first line systemic therapy for recurrent or metastatic cervical cancer. Upon study enrolment, participants will be asked to consent to the collection and storage of their archived formalin-fixed paraffin embedded (FFPE) cervical tumour tissue for future research projects. This will include fixed tissue taken at the time of primary diagnosis and cervical cancer specimens taken from any subsequent procedures prior to study enrolment. We propose to utilise this tissue to identify biomarkers predictive of benefit from maintenance olaparib and cediranib in this setting.

PARP inhibitors have been extensively investigated as maintenance treatment of ovarian cancers and studies have identified the presence of Homologous Recombination Repair Deficiency (HRD) as a predictive factor for treatment response [1 ,2]. Germline and somatic mutations in key Homologous Recombination Repair (HRR) proteins are found in a significant proportion of ovarian cancers, predominantly but not limited to BRCA1&2 [3]. The susceptibility of tumours exhibiting HRD to PARP inhibition is thought to arise as a result of sub-optimal repair of endogenous single-strand DNA (ssDNA) breaks causing an increased requirement for double-strand DNA (dsDNA) repair during cell division when the advancing replication fork converts ssDNA damage into dsDNA damage. In the absence of sufficient HRR capacity, the error-prone Non-Homologous End Joining (NHEJ) repair pathway becomes inappropriately active in cells during S phase, resulting in catastrophic DNA damage, through 'synthetic lethality' [4]. Unlike ovarian cancer, the proportion of cervical cancers with HRD has not been well studied. Analysis of cervical tumours to date does not demonstrate a significant frequency of mutations of HRR genes in cervical cancer [5, 6]. However, it is possible that cervical cancer cells may be sensitised to PARP inhibitor therapy by the acquisition of HRD by other means. It is well recognised that high-risk Human papillomavirus (HR-HPV) infection, which is associated with 99% of cervical tumours, manipulates the DNA Damage Response (DDR) at multiple junctures [7, 8], and has been shown to impact upon the efficiency of HRR in pre-clinical models [9, 10]. Differential expression levels of DDR proteins, such as FANCF, have previously been described in cervical tumours [11] and could represent a subset of cancers with increased susceptibility to PARP inhibitor therapy. The expression of PARP1 in cervical tumours has previously correlated with response to PARP inhibitors in combination with cytotoxics in recurrent cervical cancer and may also represent a potential biomarker of response [12].

The COMICE trial presents a unique opportunity to evaluate cervical tumours for markers of HRD/impaired DDR and to determine if such markers are predictive of treatment sensitivity to treatment with a PARP inhibitor/anti-angiogenic combination. It is anticipated that the Olaparib/Cediranib combination may exhibit synergism due to the induction/exacerbation of HRD in tumour cells by hypoxia. This combination demonstrated increased response rates and progression free survival (PFS) compared to olaparib alone in the maintenance treatment of ovarian cancer [13]. The mechanism by which this occurs appears to be the reduction of expression levels of key HRR proteins in hypoxic conditions [14-17] with one potential mediator of this identified as the oncometabolite (S) 2-hydroxyglutarate, which can induce PARP inhibition sensitivity in vitro [18].

While we will not be collecting on-treatment clinical samples in this study (and thus will not be able to directly evaluate anti-angiogenic therapy on tumour HRR competency and treatment response) our research group is a growing panel of primary cell lines derived from cervical tumours which will provide a model to study dynamic interactions between hypoxia, HRR efficiency and therapeutic response to olaparib in the context of HPV-associated cervical carcinogenesis. We propose a comprehensive translational research study encompassing multiple modes of molecular testing to maximise the opportunity afforded by the collection of tissue from a sizable cohort of trial participants with cervical cancer. From a planned recruitment of 108 patients to the clinical study we anticipate having consent for 96 samples of FFPE with sufficient quality/quantity of DNA/RNA for analysis. Most of these samples will be from primary diagnosis, with a much smaller number of tissue specimens from disease relapse.

i) Assessment for HRD signature in cervical tumour specimens We propose the use of DNA extracted from archival tumour FFPE taken prior to study enrolment to look for 'genomic scarring' as a marker of HRD in cervical tumours as previously described in other tumour types [19, 20]. Although commercial assays are available in this field (for example Myriad genetics 'mychoice HRD'), we propose a more cost-effective exploratory analysis in collaboration with the Centre for Genomic Research in Liverpool. This would utilise sequencing with the Agilent Sure select XT OneSeq Low resolution Backbone to assess copy neutral LOH as a marker of HRD. We expect that this will provide a superior evaluation of HRR competency than sequencing for genetic mutations in this patient population where the frequency of HRR gene mutations is expected to be low and not the primary mechanism via which HRD may be acquired. The LOH scores will then be correlated with HRR pathway protein expression, hypoxic response pathway protein expression and treatment outcome on maintenance olaparib/Cediranib (as described in sections ii-iv).

ii) Nanostring 'nCounter PanCancer Pathways' Panel Nanostring technology enables the quantitation of mRNA levels of a panel of cancer genes (770 genes), including but exclusive to DNA Damage Repair and the hypoxic response pathway. The use of this panel versus a custom panel enables maximal data accrual in a cost-effective manner, avoiding the need for custom panel development. The evaluation of expression levels of key proteins involved in these processes will enable to identification of possible mechanisms of HRD in cervical cancers and predictive biomarkers of therapeutic response. Genes of interest include ATM BRCA1 VEGFA BRCA2 FANCC VEGFC RAD51 FANCE VEGFD BRIP1 FANCF VEGFR1 FANCA FANCG HIF-1 FANCL FANCB CHEK1

iii) Immunohistochemistry will be performed on a tumour microarray (TMA) constructed from FFPE samples and correlated with findings from genomic and mRNA analysis. This will also enable direct evaluation of the expression levels of proteins of interest and their sub-cellular localisation and post-translational modification by phosphorylation. The TMA will also be used to determine the HPV status of the FFPE samples by p16 immunohistochemistry, in-situ hybridisation and RNA scope.

iv) The experimental findings will be correlated with clinical outcome as per the COMICE trial.

v) In vitro Mechanistic studies Utilisation of HPV-positive primary cell cultures for in vitro experimental work to validate findings of translational research and add to mechanistic understanding of the interaction between hypoxia and the DNA Damage Response.

vi) Blood collection Whilst the analysis of FFPE tissue is a useful tool to identify potential biomarkers of treatment response, we acknowledge that it has limitations when assessing the dynamic effects of on-treatment biological changes. There have been small prospective studies to date looking at potential circulating biomarkers for olaparib/Cediranib response in ovarian cancer which have identified some possible avenues of investigation [21]; however, they need validation in larger cohorts. We therefore propose utilising the COMICE trial as an opportunity to collect blood samples prior to treatment and at the 4 week trial visit which may be utilised in the future to further characterise circulating biomarkers of treatment benefit in a larger cohort. Tissue studies such as those described here may inform such investigations in the future. The sample size for this study is determined by the number of participants from the clinical study, which is powered for the planned outcomes of the COMICE trial. This study represents an exploratory analysis into a novel area of investigation and as such has not been subjected to statistical analysis.

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