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The pathogenesis of cutaneous squamous cell carcinoma in organ transplant recipients.

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Bullets points:

- Immunocompromised individuals such as organ transplant recipients (OTR) are at elevated risk of developing keratinocyte carcinomas (KC), particularly cutaneous squamous cell carcinoma (cSCC) and progression from normal skin through actinic keratoses, cSCC and metastasis appears to be accelerated compared to immunocompetent individuals.
- Interactions between ultraviolet radiation, dysregulated immune surveillance, direct pro- and anti-carcinogenic effects of drugs, oncogenic viruses (in particular beta genus human papillomaviruses) and host genetic susceptibility factor are likely to contribute to the pathogenesis of cSCC in OTR.
- Additional research on critical cancer drivers and the nature of the interplay between these potentially synergistic cofactors may lead to development of clinically useful prognostic and predictive biomarkers and more targeted therapeutic and preventative strategies also relevant to other immunocompromised patient populations and the general population.

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Abstract

The pathogenesis of keratinocyte carcinoma following organ transplantation is multifactorial, and recent evidence suggests a complex and often synergistic interplay between the carcinogenic effects of ultraviolet radiation, compromised immune surveillance, direct pro- and anti-carcinogenic effects of drugs, oncogenic viruses (in particular beta genus human papillomaviruses) and host genetic susceptibility factors. We present an overview of those factors for which there is currently the most convincing evidence and highlight important gaps in our knowledge. In particular, a clear understanding of the interdependence and relative contributions of these cofactors is currently lacking, yet has important implications for rational development of clinically relevant biomarkers and targeted strategies for treatment and prevention of post-transplant keratinocyte cancers.

Introduction

The immune system plays a critical role in skin cancer development, progression and response to treatment. Keratinocyte cancers (KC) in immunocompromised individuals represent a growing challenge in terms of their frequency, multiplicity and often accelerated progression. Solid-organ transplant recipients (OTR) are one of the best studied immunocompromised populations and, as summarised in the accompanying review,[1] the overall risk for cutaneous squamous cell carcinoma (cSCC) is more than 100-fold greater than the general population.[2-4] As in immunocompetent individuals (ICP), ultraviolet radiation (UVR) plays a major causal role, but other factors - altered immune surveillance, drugs, oncogenic viruses, tumour genetic/epigenetic alterations and host genetic susceptibility factors - may hold the key to explaining observed differences in epidemiology, clinical features and biological behaviour of OTR cSCC and may also contribute to the pathogenesis of cSCC in other immunodeficiencies such as HIV, chronic lymphocytic leukaemia, and immune-mediated inflammatory disorders.[5] In this review we summarise key aspects of current evidence providing insight into the influence of these factors on cSCC development and progression. We indicate where we consider further research is required to better understand their relative pathogenicity and interdependence and on how we might use this knowledge to develop future strategies for modifying their clinical impact.

Ultraviolet radiation

UVR is the principle environmental carcinogen responsible for OTR cSCC and Madeleine *et al* summarise the epidemiological and clinical evidence supporting this association[1]: more prevalent in regions of high ambient solar radiation, the majority occur on photo-exposed body sites, are more common in fair skin phototypes and those with a history of chronic UV exposure and/or episodes of childhood sunburn.[3,5,6] Photocarcinogenesis is the result of both immunomodulatory and mutagenic effects of UVR and, at the molecular level, targeted and whole exome sequencing studies in OTR cSCC confirm a high prevalence of UV-associated mutations.[7] As discussed below, certain drugs commonly used in transplantation - including azathioprine, cyclosporine, and voriconazole - may interact directly or indirectly with UVR to enhance its carcinogenic effects; human papillomavirus (HPV) may also cooperate with UVR to promote squamous carcinogenesis[5]. Improved understanding of how

these factors synergise with the effects of UV exposure at a cellular level may ultimately improve future attempts to limit or modify post-transplant UV carcinogenicity[5].

Immune surveillance and drugs used in transplantation

Reduced immune surveillance due to induction and maintenance immunosuppressive drug regimens is a major contributory factor in the increased skin cancer risk of OTRs[9]. However, establishing the differential risk conferred by individual drugs at an epidemiological level is challenging and some published data are conflicting.[1,5,9] Both duration and dose intensity may be as important as the specific drugs used: risk increases with time post-transplant;[1,3] low-dose cyclosporine is associated with a fewer cSCC;[10] CD4 counts are significantly lower in OTR with cSCC;[11] and less intensive immunosuppression in liver compared with other OTRs may account for their lower cSCC rates.[12] Such observations underpin the practice of dose reduction in OTR with KC, although the optimal timing, method and efficacy of this approach compared to switching immunosuppressive regimens has not been systematically evaluated.[13]

Direct pro-carcinogenic properties have also been identified for specific immunosuppressive drugs, the mechanisms often involving enhanced UV carcinogenicity[5,9]. Azathioprine, an inhibitor of *de novo* purine synthesis, is associated with selective ultraviolet A (UVA) photosensitivity and mutagenic effects in the skin[14-17]. The azathioprine metabolite 6-thioguanine (6-TG) replaces a small proportion of DNA guanine and becomes a strong UVA chromophore, interacting with UVA to generate reactive oxygen species which cause widespread DNA damage and protein oxidation; the latter damages the DNA repair proteome, increasing UVB mutagenicity.[14,]15 Azathioprine photosensitivity is clinically measurable[16], may be associated with a specific genetic signature[17] and can be reduced by switching from azathioprine to mycophenolate mofetil (MMF), although azathioprine metabolites may persist in the skin for several years after withdrawal.[18] MMF belongs to the same broad class of antimetabolite as azathioprine, replacing it from the mid-1990s[9]. There is a signal from cohort data that it may induce fewer cSCC,[19,20] although this may be confounded by era effects with, for example, patients receiving MMF also benefitting from greater screening and photoprotection advice compared with earlier cohorts on azathioprine. Thus, there is no clear evidence that conversion to MMF reduces tumour

accrual. Calcineurin inhibitors (CNIs, e.g. cyclosporine, tacrolimus) also demonstrate synergistic interactions with UVB and UVA[5,9] through diverse mechanisms including reduced nucleotide excision repair and apoptotic response, enhanced UVB-induced inflammation and angiogenesis, UVA potentiation of oncogenic ATF3 induction as well as UV independent mechanisms including increased TGFbeta production / signalling and suppression of p53-dependent senescence by ATF3 induction. [5,21,22]

In contrast, anti-carcinogenic effects have been observed with mammalian target of rapamycin (mTOR) inhibitors, including sirolimus (rapamycin) which was introduced in 1999.[23] Several prospective randomised trials have shown a decreased incidence of cSCC in OTRs switched from CNIs to sirolimus, an effect which is more significant if this conversion is early, specifically after the first cSCC.[24,25]. The anti-carcinogenic effects of mTOR inhibitors may be related to properties including inhibition of tumour vascularisation by suppression of VEGF; promotion of autophagy-mediated DNA repair; alterations in AKT and EGFR signalling; and promotion of memory T-cell function[5,9,26,27,28]. The greater effect on cSCC compared with BCC may relate to differential phospho-mTOR expression.[29] There have been recent concerns that benefits in skin cancer reduction are outweighed by adverse effects: analysis of 5876 OTRs from 21 randomised trials confirmed a 56% reduction in KC in OTRs receiving sirolimus, but this was accompanied by an unexplained increased risk of death.[23] However, this may reflect the generally higher doses of sirolimus used in some of the early studies included in this analysis with adverse consequences that are now avoided by lower dosing. Reassuringly, a recent retrospective study showed a reduction in skin cancer but no difference in graft rejection or survival between patients switched to sirolimus versus those not switched after post-transplant cancer.[30]

The risk of many newer immunosuppressive drugs remains uncertain and their evaluation complicated by multiple confounders, particularly duration of use. For example, belatacept, a selective T-cell co-stimulation blocker, has been used as an alternative to cyclosporine since 2011. It seems to be associated with similar skin cancer incidence in recent trials, but it is likely to be several more years before this can be confirmed.[31] Most studies reporting skin cancer have focused on maintenance rather than induction therapies and further research is needed to guide the safest induction regimes.[32]

Other drugs commonly used in transplantation may also influence skin cancer risk. Voriconazole is a triazole antifungal used in treatment and prophylaxis of invasive fungal infections such as aspergillosis. Retrospective studies have identified it as an independent risk factor for cSCC in lung transplant recipients, with multiple and aggressive tumours developing after a mean of 35 months.[33] Phototoxicity, possibly enhanced by immunodeficiency, may underpin the mechanism of action: a multistep photo-induced process with acute phototoxicity in the first year, actinic keratoses in the second/third year and cSCC by the third year onwards has been observed.[34] Moxifloxacin is a quinolone - a class of antibiotics associated with acute phototoxicity - and was recently linked to cSCC in lung transplant recipients.[35] Even more frequently used in OTRs are statins and non-steroidal anti-inflammatory drugs, classes of drugs have been linked to both increased and reduced risk of skin cancer: their risk status in OTRs remains uncertain.[36,37,38,39]

Tumour microenvironment

In addition to systemic immune dysregulation, the local tumour microenvironment plays a critical role in cancer.[40] There is evidence for a unique immune microenvironment in OTR cSCC: whilst higher numbers of circulating regulatory T cells are predictive for new cSCC development,[41] the density of tumour inflammatory infiltrate is reduced[42] and reduced CD4+ and cytotoxic CD8+ T cells infiltration, increased regulatory T cells and a blunted Th17 and Tc22 response are all predicted to lead to a 'permissive' tumour microenvironment with decreased immune surveillance[43-46]. Impaired antigen presentation (though reduced CD123+ plasmacytoid dendritic cells[43] and increased exposure to IL-22[45]) may also accelerate tumour growth and may contribute to the aggressive nature of some OTR cSCC. Most recently, immunological senescence detectable by increasing CD57 expression on circulating T cells was linked to increased skin cancer development in OTR and may represent a future predictive biomarker for cSCC risk.[46]

The mesenchymal component of the dermal compartment is also emerging as a potentially powerful driver for cSCC. Although not specifically studied in OTR, loss of mesenchymal Notch1 signalling in animal models causes stromal alterations which preceded KC formation, changes also identified in stroma adjacent to actinic keratoses/field cancerisation in human

samples and inducible by UVA.[47] Given the enhanced UVA effects in OTRs receiving both azathioprine and cyclosporine,[5,9,14-22] it is possible that these dermal effects may play a particularly important role in OTR field cancerization / cSCC and may have implications for optimising delivery and efficacy of topical chemopreventative approaches.

Human papillomavirus (HPV) infection

The most common malignancies in immunosuppressed groups are those due to known or suspected oncogenic viruses.[2] In the skin, these include Kaposi sarcoma (human herpes virus 8), post-transplant lymphoproliferative disorders (Epstein Barr Virus) and probably Merkel cell carcinoma (Merkel cell polyomavirus)[48] and a similar role for viruses in OTR cSCC has therefore been hypothesised. Human papillomaviruses (HPV) have been a particular focus of research efforts for more than three decades, driven by clinical evidence of widespread cutaneous HPV infection in OTRs, HPV-related histological features observed in some OTR cSCC and apparent clinical parallels with the rare genodermatosis epidermodysplasia verruciformis (EV) in which susceptibility to HPV infection is associated with increased risk of cSCC.[42,48-51] Current epidemiological and molecular data are supportive, but far from conclusive, and this remains an active but controversial area of OTR cSCC pathogenesis research.

HPV are small, double-stranded, non-enveloped DNA viruses that infect skin and mucosal keratinocytes[52]. More than 170 types are recognized and classified into alpha, beta, gamma, mu, and nu genera.[53]. Alpha genus mucosal HPV, principally HPV 16 and 18, play a central role in anogenital carcinoma, some head and neck SCC (HNSCC) and periungual SCC, all of which are more common in immunocompromised populations[49]. However, HPV of the beta (betaPV) rather than alpha genus have been most closely linked with cSCC.[48-51] First discovered in patients with EV, in whom they are detected in over 90% of cSCC, betaPV appear to act as co-carcinogens with UVR in EV-associated cSCC.[51] However, betaPV are also present at low level in the skin and hair follicles of more than 80% of healthy people: colonization occurs from early childhood onwards and viral load, multiplicity and seropositivity are increased by immunosuppression[48-51]. Such ubiquitous presence of betaPV inevitably poses a challenge for interpreting results of the many epidemiological studies examining a possible causal association with cSCC. Most recent analyses using state-of-the-

art techniques for detecting both the presence of HPV DNA and concordant seropositivity have shown betaPV infection to be associated with an approximately 2-fold increased risk of cSCC in OTR - of the same order as that conferred by skin phototype - although the hierarchy of specific betaPV types likely to be responsible is less consistent between studies[48-51,54] Further compelling epidemiological data are those from a recent prospective cohort study demonstrating seropositivity to betaPV at the time of transplantation is predictive for almost 3-fold increased skin cancer risk. [55]

Against the background of these complex epidemiological data, mounting evidence from biological studies is arguably more convincing and has pointed to possible synergism between betaPV and other risk factors, particularly UV[48,49,56]. The carcinogenic mechanisms of high-risk alphaPV in anogenital cancer are well established and, amongst other properties, involve inhibition by viral E6 and E7 oncoproteins of the tumour suppressors p53 and retinoblastoma (pRb), respectively[52,53]. BetaPVs generally behave differently, probably exerting pro-carcinogenic effects chiefly by enhancing accumulation of UV-induced DNA damage in the skin[51]. In normal cells, UV up-regulates cellular defence processes, ultimately leading to p53 activation, cell cycle arrest, apoptosis or DNA repair.[7,57] Expression of several types betaPV E6 and E7 (e.g. 5, 8, 38 and 49) deregulates many of these crucial pathways, mainly by directly targeting multiple transcription factors and/or transcriptional regulators, rendering infected cells highly susceptible to chromosomal instability and malignant transformation by UV.[5,48,49,51,58] For example, some betaPV E6 degrade Bak, a Bcl-2 family member which mediates UV-induced apoptosis; some also directly bind p300, the co-activator of several transcription factors including p53; HPV38 E7 induces accumulation of a p53 antagonist, ΔNp73α, which inhibits expression of several p53-regulated genes. Other pro-carcinogenic properties include abrogation of NOTCH tumour suppression, repression of TGF beta signalling, enhanced dermal invasion and evasion of host immunesurveillance.[5,49,51,56] Synergism between betaPV types and UV has been also documented in transgenic mouse models using a cytokeratin K14 promoter (K14) to drive E6 and E7 expression in the basal epidermis: in mice expressing the entire early region of HPV8 or the E6 gene alone, a single dose of UV rapidly promoted papillomas and cSCC formation[59,60] and in a K14 HPV38 E6/E7 transgenic model, UV irradiation also induced the development of actinic keratoses and cSCC.[61]

Despite these plausible functional data, the role of HPV has been directly challenged by studies in which HPV transcriptional activity in cSCC is either low or entirely absent.[49,50,56] Some researchers argue this is incontrovertible evidence that HPV infection is merely a marker of global immunosuppression and not pathogenically relevant. An alternative interpretation is the 'hit and run' hypothesis which proposes that, in direct contrast to anogenital SCC where alphaPV persistence is required for tumour maintenance, HPV is involved only in tumour initiation of cSCC by predisposing keratinocytes to UV-induced damage and, once this is achieved, the virus is not further required in tumour promotion. There is precedent for such a mechanism in bovine papillomavirus-induced oesphageal SCC (which, as in OTR cSCC, is associated with exposure to immunosuppressants present in ingested bracken) and consistent with this is the observation that betaPV viral load is higher in premalignant actinic keratoses compared with cSCC.[49-51,56]

Continued efforts to definitively clarify the role played by HPV remain a priority, given the potential implications for new developing powerful new directions for post-transplant cSCC treatment and prevention. HPV vaccination is one such strategy. Although the cornerstone of anogenital cancer prevention, existing vaccines provide type-restricted protection against alpha rather than betaPV types and so the rationale for their use in cSCC is less convincing. However, animal studies have shown that vaccination against betaPV types is a feasible approach to cSCC prevention, even in the setting of immunosuppression.[62,63] and recent experimental data hold promise that effective, next generation vaccine candidates targeting cutaneous HPV infection are in sight[64]. Although the human situation is complicated by uncertainties relating to the highest risk viruses, prevention or timely treatment of specific betaPV infections by vaccination or even antiviral drugs remains a possible future approach for reducing cSCC burden in OTR.

Genetic and epigenetics alterations

Skin tumours are amongst the most highly mutated of all human cancers due largely to the mutagenicity of both UVB and UVA and cumulative UV-exposure.[7,57] The emerging landscape of genomic alterations in cSCC underscores this high mutational burden, dysregulation of multiple signalling pathways and tumour heterogeneity.[57] Amongst studies documenting these changes, several have included analysis of OTR cSCC and, although there

appear to be some differences to ICP cSCC, none are yet identified that fully explain either the altered frequency or biological behaviour of OTR cSCC. Nonetheless, given the current pace of methodological advances, such genetic differences may yet emerge and provide clinically relevant insights into the pathogenesis of OTR cSCC, identifying new therapeutic targets and/or diagnostic and prognostic biomarkers in the process.

At the chromosomal level, studies assessing copy number aberrations in cSCC have shown high levels of genetic instability, with similar patterns across both OTR and ICP tumours.[57,65] However, some researchers suggest that this instability is lower in OTRs,[66] possibly an indication that other factors such HPV may be playing a significant role, as in HNSCC in which HPV-positive tumours have fewer genetic alterations than non-virally driven tumours. These studies would also need to stratify for additional features including cSCC differentiation (which may influence genetic instability[7,65]) and are likely to be complicated by the finding that independent OTR cSCC arising in the same individual appear to show evidence of similar allele specific imbalances, suggesting the genetic background of an individual may also influence somatic events.[67]

At the individual gene level, most OTR cSCC harbour typical UV signature mutations[7], although the existence of drug-associated mutational signatures has been suggested for azathioprine and may underscore the importance of its direct carcinogenic effects[17]. The massive mutational burden of cSCC makes it difficult to distinguish cancer 'drivers' from vast numbers of 'passenger' genetic events and critical genes have not been well defined. Nonetheless, the overall pattern emerging is of early loss of specific tumour suppressors (TP53, NOTCH1/2, CDKN2A) with subsequent involvement of diverse oncogenes, but these studies have not shown consistent differences between OTR cSCC and ICP cSCC[57].

TP53 is one of the most commonly mutated genes and occur early, already frequent and seemingly tolerated in normal skin.[68,69] *TP53* mutations were more prevalent in normal skin adjacent to OTR versus ICP cSCC in one series, although the reasons for this are not clear and possibly point to enhanced clonal expansion of UV damaged keratinocytes in OTRs, perhaps due to immunosuppressive drugs or other risk factors.[70] In contrast, 30% of OTR cSCC in another small study were negative for abnormal *TP53* immunostaining, which was

universally present in ICP tumours, and this might once again support a viral aetiology with alternative p53 attenuation through interaction of viral-host proteins.[71]

Even more frequent is mutation of NOTCH family genes.[7] A recent ground-breaking study using ultra deep sequencing of normal skin from eyelid biopsies showed an especially high prevalence of *NOTCH1* loss with biallelic *NOTCH1* inactivation found in up to 25% of normal keratinocytes.[69] This has not yet been similarly evaluated in OTR normal skin, but might prove even higher if the findings for p53 are replicated, although betaPV might alternatively attenuate NOTCH activity[56]. These findings raise intriguing questions about the gatekeeper(s) limiting progression from UV damaged skin to actinic keratoses and invasive cSCC: they may point to immune surveillance rather than specific genetic factors as critical and this may help to explain the increased prevalence and more rapid progression of cSCC in OTR and other immunosuppressed individuals.

Cancer signalling pathways in cSCC have also been evaluated in transcriptome studies and confirm RAS/MAPK pathway upregulation as characteristic of progression from actinic keratoses to cSCC in both ICP and OTR cSCC[72]. Inhibition of PI3K/AKT/mTOR activation has already proved important in secondary prevention of OTR cSCC.[24,25] Activation of transforming growth factor beta-1 (TGFbeta 1) signalling has also reported.[72] Recent data underscore its importance in driving cSCC development in general[73] and increased TGFbeta signalling has been observed in studies of OTR cSCC specifically: in the first, elevated phospho(P)-SMAD2 levels, key proteins in TGFbeta signalling, were found in both non-lesional skin and cSCC in OTR relative to non-transplanted individuals,[74] confirmed in a second study.[75] Risk factors other than UV may modulate these pathways: for example, cyclosporine interacts with TGFbeta signalling and the effects of sirolimus on mTOR signalling may in part explain its effects in secondary prevention of OTR cSCC[9].

Epigenetic changes in cSCC, including DNA methylation, histone acetylation and the activity of microRNAs, represent important targets for possible therapeutic intervention although published data remain limited.[57] OTR cSCC have been included in a few studies only: p16 methylation was found to be higher in ICP cSCC and miR-135 expression was similar in both OTR and ICP cSCC[77], but this is an area where further research should particularly focus

given that, compared with genetic mutations, epigenetic alterations are generally more tractable therapeutic targets.

Finally, in germs of genetic susceptibility, as discussed by Madeleine et al, germline single nucleotide polymorphisms (SNPs) may be associated with increased cSCC risk, although only a few studies have been conducted in OTR and as such they cannot yet be used for risk prediction.[1]

Conclusions

In this review, we have highlighted key data underpinning current understanding of factors influencing the pathogenesis of cSCC arising in OTR (Figure 1a). The striking increase in cSCC incidence and the accelerated progression from sun exposed skin, to actinic keratoses, cSCC and metastasis experienced by OTRs emphasises how critical an intact immune system is to both pathogenesis and clinical behaviour of cSCC. Unravelling the relative importance and complex interdependencies of UV, immune surveillance, pro- / anti-carcinogenic drugs effects and beta-papillomaviruses, genetic alterations and host genetic susceptibilities remains a challenging task, yet will undoubtedly be essential for future development and optimisation of rational therapeutic and preventative interventions (Figure 1b).

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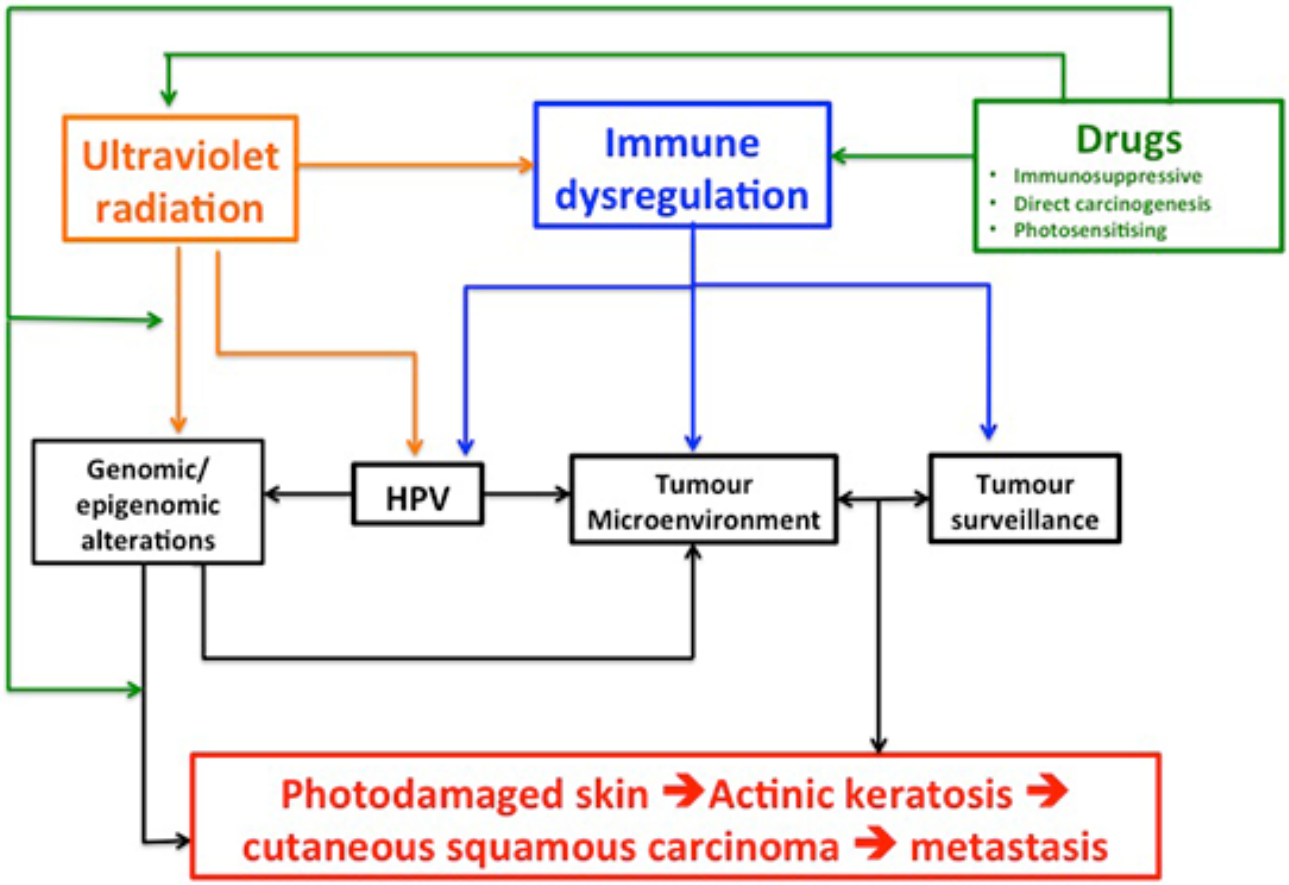

Figure 1: Summary of the complex interplay of multiple influences in the development and progression of cutaneous squamous cell carcinoma in OTR