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Review

Ubiquitination and the Ubiquitin – Proteasome System in the Pathogenesis and Treatment of Squamous Head and Neck Carcinoma

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Abstract. *Squamous carcinomas of the head and neck area are carcinomas that were traditionally associated with alcohol and tobacco abuse. More recently, a pathogenic relationship of oncogenic human papilloma viruses (HPV) with head and neck cancer of the oropharynx and the base of the tongue has been revealed. Two proteins of HPV, E6 and E7, are involved in neoplastic transformation not only in the head and neck but in other locations, where these epitheliotropic viruses cause carcinomas, such as the uterine cervix and the anal region. The E6 viral protein associates with cellular E3 ubiquitin ligase E6-AP and promotes degradation of tumour suppressor p53 by the proteasome. This molecular event reveals the important role that the ubiquitin-proteasome system (UPS) plays in the pathogenesis of head and neck cancer. The role of this system in head and neck carcinogenesis is not restricted to the destruction of p53 but extends to most, if not all, signaling pathways that regulate carcinogenesis in this location. These roles are reviewed here and implications for treatment are discussed.*

Head and neck carcinoma is one of the leading causes of cancer death and includes squamous carcinomas of the oral cavity, the pharynx and the larynx. Despite progress achieved in the past several years by the introduction of combined chemoradiation therapy for its treatment, head and neck squamous carcinoma remains a difficult disease to treat, especially when locally advanced or metastatic (1). Classically, head and neck cancer was considered a disease associated with alcohol and tobacco use, with most patients being heavy users of both substances. More recently, an additional pathogenic association has been revealed, the one of human papillomaviruses (HPV) with squamous head and neck carcinomas, especially with those localized in the tonsils and the base of the tongue (2). Thus, there are currently two major subtypes of head and neck cancers based on pathogenesis and clinicopathological characteristics: the classic alcohol and tobacco-related and the more recently identified viral-related. Tobacco and alcohol-related head and neck carcinomas have no predilection for site, are mostly seen in older patients with long exposure to the causal agents, frequently harbor *p53* mutations, and are currently decreasing in incidence (3). Viral-related head and neck carcinomas are seen in younger patients, have a predilection for the oral cavity and base of the tongue, harbor wild-type *p53* and are increasing in incidence (3). HPV-associated head and neck carcinomas probably have a better prognosis than other types of squamous head and neck cancers and respond better to treatments (4). HPV exerts its carcinogenic action by disabling two key cellular tumour suppressor pathways regulated by p53 and retinoblastoma (Rb) proteins through the expression of two viral proteins E6 and E7, respectively. The mechanism of action of E6 relates to its association with cellular Homologous E6-AP Carboxy-terminal (HECT) domain ligase E6-associated protein (E6-AP) which promotes p53 ubiquitination and degradation by the proteasome. This mechanism brings ubiquitination and the ubiquitin-proteasome system (UPS) to the front stage of viral carcinogenesis. Several key factors of both virally- and non-virally-induced head and neck carcinogenesis are regulated by this system and are discussed in the following sections.

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Brief Overview of Molecular Carcinogenesis of Squamous Carcinomas in the Head and Neck

HPV-related head and neck carcinomas represent a significant minority of these types of cancer and probably account for about one in five cases of head and neck cancer in different ethnic populations (5). As mentioned, two viral proteins E6 and E7, are important for cancer promotion. Carcinogenic subtypes of HPV are the same for head and neck cancer and uterine cervical cancer and pathogenic mechanisms are shared. Briefly, E6 favours p53 proteasome degradation by facilitating its ubiquitination through the action of E3 ligase E6-AP. This is a HECT family ligase (see next section) which has taken its name precisely from this interaction with E6 (6). Tumour suppressor p53 is one of the most frequently mutated proteins across different types of cancers and these mutations debilitate p53 function as apoptosis inducer, cell-cycle regulator and guardian of genome integrity (7). In HPV-related cancer, p53 retains its wild-type status but instead is functionally disabled through untimely protein degradation. Viral protein E7 interferes with another carcinogenesis regulator and tumour suppressor, the Rb gene product (8). It associates with Rb and prevents the inhibitory interaction of Rb with transcription factor E2F-1, a key element in the progression of the cell cycle from the G_1 to the S phase. As a result of the inability of Rb to interact with E2F-1, the transcription factor is freed to transcribe target genes which contribute to cell-cycle progression (9).

Additional cellular targets emerge in HPV viral carcinogenesis and include for example proteins involved in cell polarity such as Scribble, PALS1-associated tight junction protein (PATJ), and E-cadherin targeted for degradation with the aid of E6 (10, 11).

In head and neck carcinogenesis not related to viruses, mutations of p53 are present in the majority of cases (12). These are mutations that lead to disabling of the transcriptional function of p53, with a resulting dysfunction of the pathways of apoptosis or cell-cycle arrest induced by the transcription factor after DNA damage or other stresses. p53 mutants additionally possess gain of functions that promote carcinogenesis beyond the absence of the wild-type protein (13). In head and neck cancer, mutant p53 promotes mitotic entry by inducing genes such as cyclins A and B (14).

In HPV-independent head and neck carcinogenesis, Rb tumour suppressor is frequently functionally inactivated due to mutations or promoter methylation of upstream cyclin dependent kinase (CDK) inhibitor p16 located at chromosome 9p21, or amplifications of *cyclin D1* gene located at chromosome 11q13 (15, 16).

Growth signals received at the cell surface by growth factor receptors are mainly transduced inside the cell through the rat sarcoma viral oncogene homolog (RAS)/RAS-activated factor (RAF)/MAP-ERK kinase (MEK)/extracellular signal-regulated kinase (ERK) proteins and the phospho-inositide-3-kinase (PI3K)/v-akt murine thymoma viral oncogene homolog (AKT) kinases. Head and neck cancer cells gain growth advantage by up-regulating these pathways in multiple ways. Genes for tyrosine kinase receptors epidermal growth factor receptor (EGFR) and MET (also known as scatter factor) at the short and long arms of chromosome 7, respectively, are frequently affected in head and neck cancer (17, 18). In a significant minority (up to 30%) of cases *EGFR* may be amplified and the protein overexpressed. The same is true, in some cases, for *MET*. Additional lesions are present in the PI3K/AKT branch of signal transduction in the form of either amplification or mutation of the catalytic subunit of PI3K kinase, *PIK3CA* or deletions or inactivating mutations of protein phosphatase and tensin at chromosome 10 (PTEN), an inhibitor of AKT kinase activation (19, 20).

Another important pathway with a more complex role in carcinogenesis is the one of transforming growth factor-β (TGFβ) (21). TGFβ ligates two cell surface receptors called TβRI and TβRII and transmits signals through a canonical route involving activation of human mothers against decapentaplegic (SMAD) transcription factors. Alternative signaling occurs through activation of PI3K and MAPK pathways with the co-operation of TNFα receptor-associated factor 6 (TRAF6) and TGFβ-activated kinase 1 (TAK1) (22). Activated TβRII may also phosphorylate polarity complex protein partitioning defective 6 (PAR6) which then promotes degradation of RhoA GTPase by recruiting E3 ligase SMAD-specific ubiquitin ligase 1 (SMURF1), leading to dissolution of tight junctions. TGFβ signaling is tumour-suppressive in pre-malignant cells but encourages tumour propagation in advanced malignancies and participates in the epithelial to mesenchymal transition (EMT) program that promotes invasion and metastatic spread of epithelial cancer (21). These conflicting actions are dependent on parallel pathways concomitantly activated and the general cellular context.

Although the pathways discussed above play distinct roles in signaling, there is significant cross-talk between them and an amplitude of parallel regulations, making dysfunction in any one of them affecting the others. A simplified view of the cross-talk of head and neck cancer-affected pathways is presented in Figure 1.

Ubiquitination and the UPS. Ubiquitination (also called ubiquitylation) is a post-translational modification of proteins that consists in the attachment not of a chemical unit such as is the case with, for example, phosphorylation or acetylation, but of an entire protein, ubiquitin. Initially thought to serve degradation of defective proteins, ubiquitination has now been proven to be very versatile and

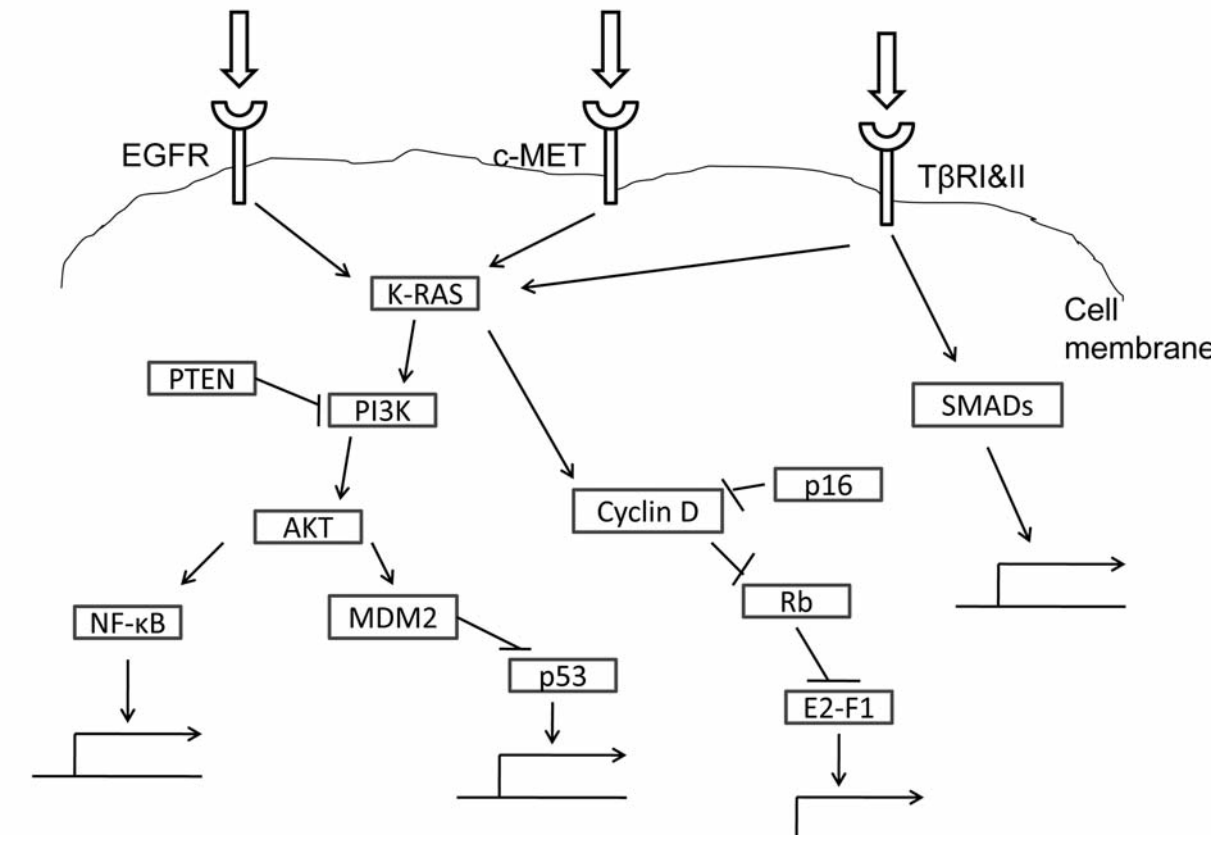

Figure 1. *Simplified overview of pathways involved in head and neck carcinogenesis.*

involved in the regulation of virtually every cellular process (23). Ubiquitin is a 76-amino-acid protein with a high concentration in cells and significant sequence conservation between species. It has lysine residues at positions 6, 11, 27, 29, 33, 48 and 63. Links can be formed through any of these lysines or through the amino-terminal methionine of ubiquitin (24). Attachment to a target protein is a strictly regulated process and is executed with the catalytic action of three types of enzymes (Figure 2). An ATP-dependent enzyme called E1, or ubiquitin-activating enzyme, is charged with the first step. It binds and activates ubiquitin and transfers it to an ubiquitin-conjugating enzyme also called E2. E2-linked ubiquitin is transferred to a target protein by a ubiquitin ligase, or E3 enzyme. Subsequent attachment of additional ubiquitin molecules onto the first attached molecule leads to the creation of poly-ubiquitin chains. A target protein may also be ubiquitinated in multiple residues (multi-ubiquitination). In the human genome, there are two E1 enzymes, about 30 to 40 E2 enzymes, and about 600 E3 ligases (25) (Figure 2).

Besides ubiquitin, there are several ubiquitin-like cellular proteins such as small ubiquitin-like modifier (SUMO), neuronal precursor cell-expressed developmentally down-regulated 8 (NEDD8) and interferon-stimulated gene 15 (ISG15). All these proteins use attachment cascades and enzymes similar to ubiquitin and play specific roles in cellular functions (26). Diverse roles in carcinogenesis have also been described but will not be further discussed here.

The two ubiquitin-activating E1 enzymes in the human genome are called ubiquitin-activating enzyme-1 (UBE1) and ubiquitin-like modifier-activating enzyme-6 (UBA6) (27). UBE1 works uniquely with ubiquitin, while UBA6 serves, besides ubiquitination, for the attachment of the ubiquitin-like protein human leukocyte antigen F-associated transcript 10 (FAT10). Other ubiquitin-like molecules are served by distinct E1s (28). E1 enzymes can recognize their cognate molecule and exclude others by specific structural interaction surfaces. The same is true for recognition of their cognate E2 enzymes.

E2 enzymes are situated in the middle of the ubiquitination cascade receiving activated ubiquitin from E1 and transferring it to E3 or directly to the target protein with facilitation by an E3 ligase. About three dozen E2s exist in the human genome and are characterized by a ubiquitin-

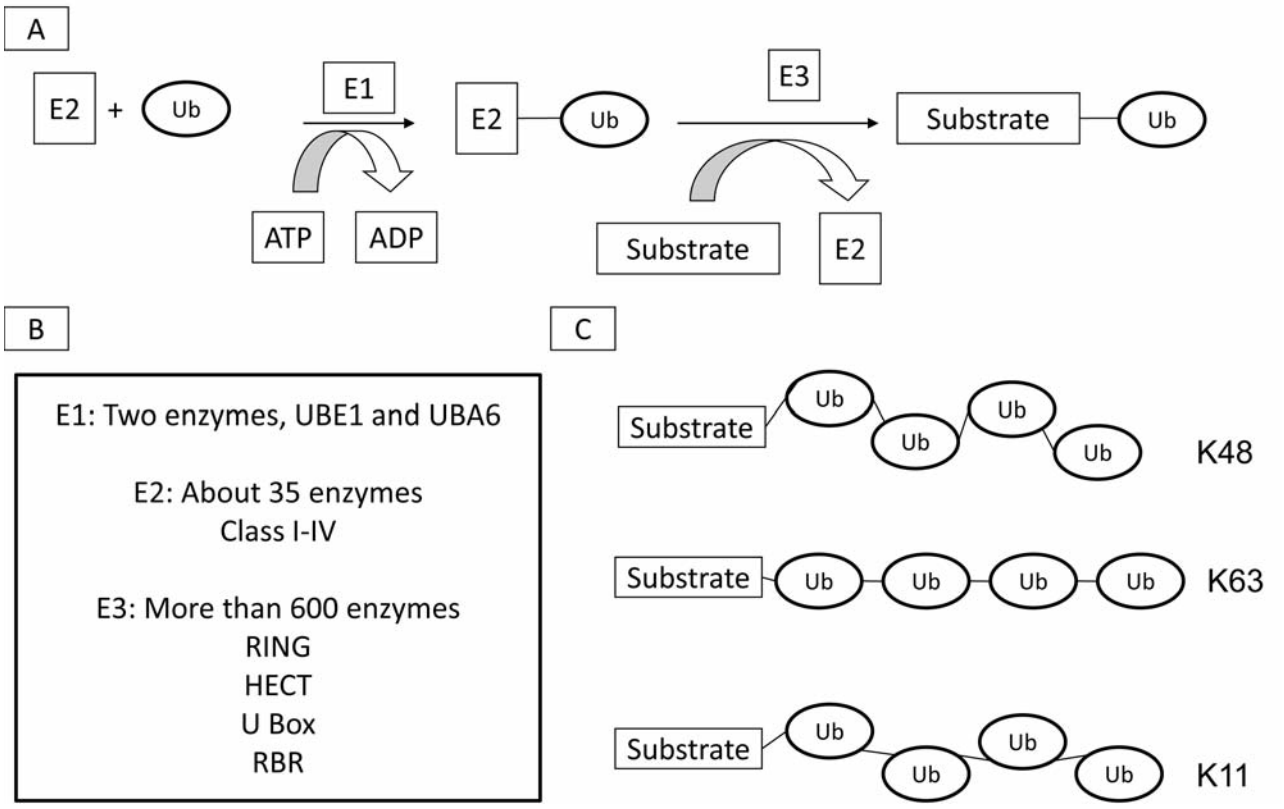

Figure 2. *A: Schematic representation of the ubiquitination cascade. B: Recapitulation of ubiquitination cascade components. C: Schematic representation of types of ubiquitin chains. K48 chains are involved in general proteasome recognition and degradation of substrates, K63 chains are involved in endocytosis and lysosome degradation and K11 chains are taking part in specific substrates degradation during the cell cycle.*

conjugating domain of about 150 amino acids, which includes the catalytic cysteine receiving the ubiquitin molecule (29). Some E2 enzymes are composed of just this domain while others bear amino-terminal and carboxy-terminal extensions contributing to protein interactions with E3 ligases or substrates (30).

The two major types of E3 ligases, really interesting new gene (RING) type and HECT type, differ in their mode of catalysis but both execute ubiquitin ligation to a target protein. RING type E3s act by bringing E2-bound ubiquitin in proximity with the substrate protein, facilitating ubiquitin transfer to the substrate. Besides physically abridging E2s with substrates, RING E3s probably mediate a conformational change of bound E2 that contributes to ubiquitin transfer (31). In contrast, HECT-type E3s possess an active cysteine residue that covalently binds ubiquitin before transferring it to the substrate. A third type of E3 ligase called U-box domain ligase is considered a sub-type of RING E3s because U-box domain has a RING-like conformation and the mechanism of action is also by bridging E2-bound ubiquitin with the substrate, similarly to RING type E3s. A more recently discovered type of ubiquitin ligase is the RING between RING (RBR) type, with a somewhat hybrid mode of function (32). RBR type E3s feature a typical RING domain which conducts ubiquitin-loaded E2s, followed by a domain called in between RINGs (IBR). In the carboxy-terminal side of the IBR domain there is an atypical second RING domain incorporating an active cysteine. This cysteine accepts ubiquitin from E2 in a much similar way to that of the active cysteines of HECT-type E3s, forming a thiol-ester bond. RING-type E3s are by far more abundant and comprise about 95% of human E3s (33), while HECT-type E3s number 28 members in human genome (6) and RBRs 12 members (32).

Attachment of a ubiquitin or multiple ubiquitin molecules to a target protein results in different outcomes depending on the lysine that mediates attachment. Lysine 48 ubiquitin chains of at least four molecules are recognized by the proteasome and signal for degradation of the target protein (25). Occasionally, lysine 6- and 11-mediated ubiquitin chains have been observed to also signal for target protein proteasome degradation, notably, in the case of lysine 11, in cell-cycle regulation (34). Lysine 63-

mediated ubiquitin attachment leads less often to proteasome degradation but serves in autophagy-mediated proteolysis, as well as to non-proteolytic functions such as DNA repair and receptor kinase endocytosis (35). Ubiquitination also participates in various other cellular processes such as, for example, angiogenesis, DNA transcription, DNA damage tolerance and establishment of epithelial cell polarity.

Similar to other post-translational modifications, ubiquitination is reversible and covalently attached ubiquitin molecules can be removed by de-ubiquitinating enzymes which preserve cellular ubiquitin pools, reverse inappropriate ubiquitination and dynamically regulate processes in which ubiquitination participates. There are several dozen de-ubiquitinating enzymes in the human genome that belong to five distinct families. Enzymes of four of these families are cysteine proteases and the fifth family comprises of zinc metalloproteases (36). De-ubiquitinizing enzymes mostly antagonize ubiquitination processes but at times may also promote them by reversing auto-ubiquitination of E3 enzymes, an example being de-ubiquitinase herpesvirus-associated ubiquitin-specific protease (HAUSP, also known as USP7) and E3 ligase mouse double minute 2 (MDM2), as will be discussed later (37).

The proteasome or 26S proteasome is a hollow cylinder-shaped multiprotein organelle of 2.500 kDa comprising a core particle (CP or 20S proteasome) flanked on two sides by a regulatory particle (RP or 19S proteasome). It is found in both the cytoplasm and the nucleus (38). RP structurally has two parts, a lid and a base that contacts the CP. It functions in the recognition of the ubiquitinated protein marked by lysine 48 chains and denaturing of the protein. Protein components of RP have de-ubiquitination activity, which allows ubiquitin molecules to be recycled. Finally, RP base proteins with ATPase activity propel the target protein to the CP (39). CP is made up of four rings of seven-member proteins, each making contact with the neighbouring ring. The two rings in the external part of CP are identical and are called α rings (sub-units named α1 to 7) and the two central rings are also identical and are called β rings (with sub-units β1 to 7). The three enzymatic activities of the proteasome reside in the central rings of the CP. These include a trypsin-like (post-basic residue cleavage) activity, a chymotrypsin-like (post-hydrophobic residue cleavage) activity and a post-glutamyl (caspase-like or post-acidic residue cleavage) activity that are conferred by sub-units β1, β2 and β5, respectively, and may degrade almost any target protein, producing fragments of 4 to 14 amino-acids (40).

Regulation of Molecular Pathways of Head and Neck Cancer by Ubiquitination and the UPS

Pathways participating in the pathogenesis of head and neck carcinomas are regulated by ubiquitination and the UPS. Some of these regulations are well-understood and new ones are continuously being discovered.

Altered regulation of p53 by ubiquitination as mentioned, has brought into the spotlight, the importance of UPS in HPV viral carcinogenesis. p53 is a transcription factor that has evolved to protect the genome of adult cells (41). It does so by being activated after DNA damage and mediates either apoptosis or cell cycle arrest. Under baseline conditions, in the absence of cell stress, p53 is unstable and kept under control by E3 ligase MDM2 which ubiquitinates it, leading to degradation by the proteasome. Although MDM2 auto-ubiquitinates itself, under baseline conditions, the de-ubiquitinase HAUSP binds it and reverses this ubiquitination, stabilizing MDM2 (42). Other E3 ligases have also been shown to perform the same function of p53 degradation, bearing witness to the importance of the strict regulation of p53 (Figure 3). DNA damage response involves activation of kinases such as ataxia telangiectasia mutated (ATM) and ataxia telangiectasia related (ATR) which then activate checkpoint kinases CHK1 and 2 (43). These kinases, in turn, phosphorylate p53, leading to its stabilization and activation. Concomitant phosphorylation of MDM2 by checkpoint kinases leads to decreased association with HAUSP and finally results in MDM2 destabilization through auto-ubiquitination (44). When activated in response to DNA damage, p53 executes a transcriptional program leading, depending on post-translation modifications and co-activators available, to either cell-cycle arrest which gives time for DNA repair, or to apoptosis if damage is sensed as being irreversible.

Beyond its role in cell-cycle inhibition and apoptosis, p53 is an EMT suppressor and, as a result, a suppressor of metastatic potential of neoplastic cells. This function is mediated through induction of microRNAs of the miR-200 and miR-192 families which then suppress translation of EMT-inducing transcription factors ZEB1 and 2 (45, 46). In addition, other transcription factors promoting EMT such as SNAIL, SLUG (also called SNAIL2) and TWIST are up-regulated in cancer cells when p53 is knocked-out and cells undergo EMT (47). p53 down-regulates SLUG through promotion of its MDM2-mediated ubiquitination and degradation by the proteasome. This down-regulation allows E-cadherin expression and stabilization of adherens junctions (48).

Rb is another protein targeted by HPV carcinogenesis in association with p53. Rb is an inhibitor of transcription factor E2F-1, a critical factor for the progression of cell cycle from the G_1 to S phase and a part of the cyclin D/CDK4/6 /Rb/ E2F-1 axis. Physiologic mitogenic signals activate cyclin D which in a complex with CDKs 4 or 6 phosphorylate and inactivate Rb. Transcription factor E2F-1 is freed to transcribe genes promoting cell-cycle progression (49). A feedback loop exists which favours expression of CDK inhibitor $p16^{INK4A}$ and inhibits CDK4/6 in an attempt

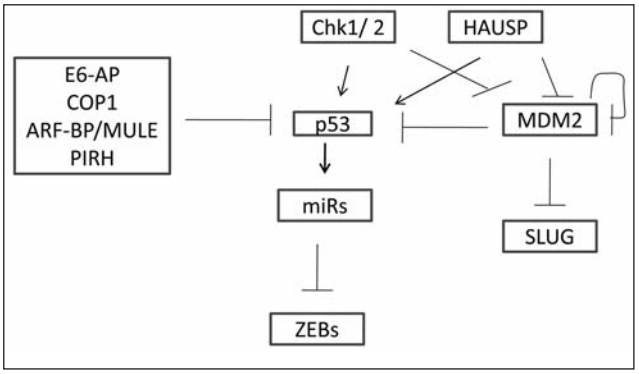

Figure 3. *Interplay of phosphorylation and ubiquitination in the balance of p53 and mouse double minute 2 (MDM2). Activation of checkpoint kinases (CHK) in stress phosphorylates both p53 and MDM2 leading to increased binding of de-ubiquitinase herpesvirus-associated ubiquitin specific protease (HAUSP) to p53 and decreased binding to MDM2, finally resulting in increased p53 stability. Other ubiquitin ligases such as E6-associated protein (E6-AP) (particularly relevant in HPV-dependent carcinogenesis), ARF-binding protein 1/ Mcl1 ubiquitin ligase E3 (ARF-BP/ MULE) and p53-induced RING H2 (PIRH2) regulate additionally the p53 ubiquitination state. The p53/ MDM2 system bears special weight for epithelial–mesenchymal transition (EMT) and metastasis as both zinc finger E box-binding homeomeobox (ZEBs) and SLUG transcription factors are regulated by it.*

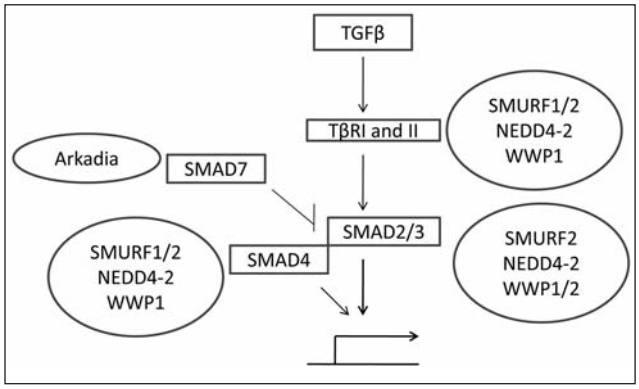

Figure 4. *Core transforming growth factor-β (TGFβ) signaling components and regulation by the UPS. TGFβ activates serine threonine kinase surface receptors TβRs which then activate receptor human mothers against decapentaplegic (R-SMADs), SMAD2 and 3. Binding of R-SMADs to co-SMAD SMAD4 promotes transcription from SMAD-binding elements (SBEs). I-SMAD, SMAD7 inhibits the cascade. E3 ligases that regulate each component are depicted in ovals besides it.*

to shut down the pathway. HPV protein E7 sequestrates Rb, activating E2F-1. This activation, besides promotion of cell proliferation, leads to an increase of $p16^{INK4A}$, a fact that is exploited in the diagnosis of HPV-positive cancers, which are positive for $p16^{INK4A}$ by immunohistochemistry (50). In HPV-independent head and neck cancer, the cyclin D/CDK/Rb pathway is also affected by frequent lesions such as $p16^{INK4A}$ promoter methylation or gene deletions (15), and *cyclin D* gene amplifications (51). It is interesting to note that $p16^{INK4A}$ gene deletions would also affect p53 activity because p53 activator $p14^{ARF}$ is transcribed from the same locus at chromosome 9p21 with an alternative reading frame (52). Ubiquitin ligase MDM2 provides another link between p53 and Rb pathways, being a target gene of p53 and ubiquitinating both p53 and Rb for proteasomal degradation (53). On other occasions, Rb interaction with MDM2 leads to cell differentiation (54). Transcription factor E2F-1 is regulated by the UPS in a cell cycle dependent manner. The E3 ligase involved this time is S-phase kinase-associated protein (SKP2) that promotes E2F-1 proteasomal degradation in the end of S phase (55). Cyclin D is another component of the pathway with a short half life whose stability is regulated by ubiquitination although the E3 ligase(s) involved in this instance remains debatable (56).

Both p53 and Rb pathways are involved in viral and non-viral head and neck carcinogenesis and both are regulated in multiple levels by ubiquitination and the UPS. Although the specific lesions are different in the two cases, the end effects are similar, promoting cancer cell survival and proliferation. Interestingly, at least in some cellular contexts, loss of Rb leads to p53-induced apoptosis (57, 58) and this may explain the need for concomitant *p53* mutations or neutralization in head and neck cancer where dysregulation of the Cyclin D/CDK/Rb axis is part of the neoplastic process.

TGFβ signaling plays a complex role in carcinogenesis, acting as a tumour suppressor during initial cancer development and having a tumour-promoting role associated with EMT, invasion and metastasis in more advanced cancer when the RAS /MAPK pathway is activated in parallel and p53 is disabled due to mutations or functional inactivation (59). Duration of TGFβ signal and the timing of this signal in regard to the cell-cycle phase that a given cell transverses may be additional factors in determining TGFβ signaling outcome (60-62). TGFβ is stored in the extracellular matrix in a latent form and when released binds its cell surface serine/threonine kinase receptors TβRI and TβRII (63). In the canonical TGFβ signaling pathway, after ligation, receptors phosphorylate and activate the receptor SMAD proteins (also called R-SMADs) SMAD2 and 3. Phosphorylation of SMAD2/3 creates a binding site for co-SMAD, SMAD4 and the complex moves to the nucleus where it acts as transcription co-factor, recognizing SMAD-binding elements (SBEs) on DNA with the consensus sequence CAGAC (21). TGFβ may also signal through pathways that do not involve SMADs and are referred to as non-canonical. For example, TβRI activates RAS through adaptor protein src homology and collagen homology A (SHCA), and proteins son of sevenless 1 (SOS1) and growth

factor receptor-bound protein 2 (GRB2) (64). TGFβ activation of the RAS pathway promotes EMT, by inducing transcription factors SNAIL, SLUG and ZEB1 and 2, resulting in E-cadherin suppression (65, 66), an event also depending on neutralization of p53 by MDM2 induction (67, 68). Another non-canonical TGFβ signaling pathway further promotes EMT by a transcription-independent mode, through phosphorylation of the polarity complex protein partitioning defective (PAR6) by TβRI. Phosphorylated PAR6 recruits E3 ligase SMURF1, promoting degradation of exchange factor RhoA and actin de-polymerisation, leading to tight junction disassembly (69).

All backbone components of the TGFβ cascade, including TβRI and TβRII and SMADs, are regulated by ubiquitination and proteasome degradation (70). Members of the NEDD4 family of HECT E3 ligases such as NEDD4-2, SMURF1 and 2, WW domain-containing protein 1 (WWP1) and itchy protein E3 ubiquitin ligase (ITCH/AIP4) participate in this regulation (71) (Figure 4). In addition ubiquitination may lead to non-degradative outcomes such as enhanced interactions with partner proteins (72). For example, SMAD2 ubiquitination by ligase Itch/AIP4 promotes interaction and phosphorylation of SMAD2 by TβRI, activating the cascade (73). In head and neck cancer, TGFβ signaling is frequently down-regulated due to deletions or mutations of the receptors or SMAD2 and 4 (74, 75). SMAD4 mutations are frequent in various types of cancer and produce proteins that are more prone to ubiquitination and degradation than the normal isoform (76). In addition, mice with knock-out of SMAD4 in their mouth epithelium develop squamous carcinomas (77). Thus it appears that in head and neck carcinogenesis, as in other types of cancer, inactivation of TGFβ signaling by SMAD4 mutations, which may render it more prone to proteasome degradation favours cancer progression. This may be related to the fact that head and neck cancer cells harbour a dysfunctional CDK4/6/Cyclin D/Rb/E2F-1 pathway as discussed above and bypass the G_1/S phase checkpoint. In this background, TGFβ signaling may favour apoptosis instead of EMT (62) and, as a result, its impediment levies this brake for carcinogenesis.

Tyrosine kinase receptors including EGFR, c-MET and neurotrophic growth factor receptor (NGFR) and their downstream pathways are implicated in head and neck carcinogenesis. Two main downstream pathways are activated by these receptors, the RAS/RAF/MEK/ERK pathway and the PI3K/PTEN/AKT pathway (78). The former culminates to activation of AP-1 family transcription factors while targets of PI3K/AKT phosphorylation include apoptotic members of BCL2 family which are inhibited, E3 ligases MDM2 and F box and WD domain-containing ligase (FBW7) which are protected from inhibition, apoptotic transcription factor F box O (FoxO), glycogen synthase kinase 3β (GSK3β), and caspase-9, which are all inhibited (78, 79). The mammalian target of rapamycin (mTOR) cascade is also activated by AKT leading to enhanced cell growth and protein production (80). In HPV-associated head and neck carcinogenesis, viral E6 protein promotes mTOR activation by favouring E6AP-mediated degradation of its inhibitor tuberous sclerosis complex-2 (TSC2) (81). Increased mTOR activity results in increased translation of E7 viral protein further supporting the neoplastic process (82). The janus kinase (JAK)/signal transduction and activator of transcription (STAT) pathway may also become activated by tyrosine kinase receptors and activation of STAT3 transcription by EGFR signaling, for example, co-operates with it to induce more aggressive cancer with EMT features (83).

As mentioned, lesions expressing EGFR and c-MET are present in a significant minority of head and neck cancers (84, 85). Indeed, EGFR-targeted therapy with the monoclonal antibody cetuximab is the only such therapy approved and used clinically for this type of cancer (86). In addition, the wild type form of tyrosine kinase B (TRKB), the surface receptor for brain-derived neurotrophic factor (BDNF) and the ligand are expressed in the majority of head and neck carcinomas, in contrast to the normal upper aerodigestive epithelia (87). AKT is activated downstream of TRKB and promotes motility and invasion of head and neck squamous carcinoma cells (87). In HPV-dependent carcinogenesis, viral E7 protein augments the tumorigenic activity of another receptor tyrosine kinase pathway, that of insulin-like growth factor receptor (IGF-R) by binding and promoting proteasome degradation of inhibitor IGF-I binding protein-3 (IGFBP-3) (88).

Tyrosine kinase receptor pathways are regulated by the UPS in multiple ways. Several core components of these pathways are proteasome substrates. Examples include kinases RAF (89), ERK1 and -2 (90) and -3 (91) of the RAS branch and the regulatory subunit p85 of PI3K (92) and kinase AKT (93) of the PI3K/AKT branch. Additionally components of the JAK/STAT pathway are UPS-regulated (94). Tyrosine kinase receptors themselves are regulated by ubiquitination after ligand binding. Ligand binding induces ubiquitination with the aid of E3 ligase casitas B lineage lymphoma (CBL) which then mediates clathrin-dependent receptor endocytosis through recognition by ubiquitin-binding domains in clathrin-associated proteins of clathrin-coated pits (95). Receptor endocytosis may lead to receptor degradation or recycling to the cell surface in order to be available for further ligand interactions. In other instances signaling may even continue from internalized receptors in the early endosomes. Oncogenic mutations of tyrosine kinase receptors may not only increase the activation of receptors but also promote their surface recycling (96).

Phosphatase PTEN is an inhibitor of PI3K activation and is mutated in a subset of squamous head and neck carcinomas (20). It also constitutes an additional element of pathways emanating from surface kinase receptors that is regulated by the UPS. HECT domain ligase NEDD4 is the E3 enzyme involved in the poly-ubiquitination of PTEN that leads to its proteasomal degradation (97), although it may not be the only E3 ligase involved in this degradation or it may even not be involved at all in this function in some cellular contexts (98). In addition to poly-ubiquitination, PTEN may also become mono-ubiquitinated, a modification that does not promote degradation but nuclear entry (99). Inside the nucleus, PTEN functions in protection of chromosome integrity by promoting centromere stability and repair of DNA double-strand breaks. Given these tumour-suppressing effects of PTEN, its negative regulator NEDD4 may act as an oncogene in cellular contexts where it is inadvertently up-regulated and PTEN is functional.

Nuclear factor of κ chains in B lymphocytes (NF-κB) represents a family of five transcription factors that are important for inflammation, immunity and carcinogenesis (100). Although NF-κB components are not directly mutated or amplified in squamous head and neck carcinomas, the NF-κB transcription system is situated downstream of several of affected pathways in these types of cancer and hence it co-operates in carcinogenesis. Moreover it is regulated by UPS (101). Similar to PI3K/AKT pathway, NF-κB has been associated with chemotherapy resistance in various types of cancer (102). It is also a downstream target of AKT kinase which phosphorylates NF-κB, activating I-κB kinase (IKK). IKK phosphorylates NF-κB inhibitor I-κB which is then ubiquitinated by E3 ligase β transducin-containing protein (βTRCP) for proteasome degradation. NF-κB is activated by several other pathways including TNFα receptor, receptor of activated NF-κB (RANK) and diverse cytokine receptors. It may also become activated directly by DNA damage (103). NF-κB inhibits apoptosis by inducing genes such as *BCL2*, *BCL-XL* and *A1* and promotes proliferation by inducing cyclin D1 and c-MYC. Among NF-κB target genes is also interleukin 6 (*IL6*), which results in more aggressive head and neck cancers through JAK-STAT3 signaling (104). In addition, NF-κB is involved in tumor progression and metastasis through induction of genes of the core EMT program such as *SNAIL* and *SLUG*, *TWIST* and *ZEB* (105). Several other ubiquitination events regulate NF-κB activity. For example, NF-κB modulator BCL3 is regulated by the UPS, and given that it is an inhibitor of p53 transcription activity (106), it integrates signals for both transcription factors, with UPS serving as a critical node.

In addition to K48 chains added to I-κB by ligase βTRCP and leading to its proteasome degradation, modification with ubiquitin chains of other types is important in NF-κB signaling (107). K63 chains serve as docking sites for the assembly of the receptor complexes that lead to NF-κB activation (101). Other types of chains including linear chains (M1 linked) and K11 have novel but less well-defined roles (107). Several de-ubiquitinating enzymes including cylindromatosis syndrome D (CYLD), cezanne, A20, USP11 and USP15 are also involved in NF-κB signaling and help fine-tune outcomes (108).

In head and neck cancer, besides being activated by pathways activating kinase AKT, NF-κB is positively influenced by TGFβ disabling (109). A role of mutations or decreased activity of wild-type p53 in this TGFβ disabling by down-regulating of different TβRs has been suggested. Reciprocally, NF-κB contributes to shutting down any residual p53 activity by inducing ligase MDM2 (110). Up-regulation of NF-κB by AKT, TGFβ disabling and the highly inflammatory environment present in head and neck carcinomas induce cyclin D and perturb the G_1 to S cell-cycle checkpoint (111), an event that may be reversed by NF-κB inhibition (112). NF-κB may also induce chemokine CXCR4 (113, 114). This chemokine and its ligand CXCR12 promote movement and invasion of cancer cells. In agreement with a role of NF-κB in head and neck carcinoma invasion and metastasis, a higher percentage of lymph node metastases and their associated primary tumours of patients with head and neck cancer expressed nuclear NF-κB compared with patients with no lymph node metastases (115).

Therapeutic Perspectives of Ubiquitination and the UPS in Head and Neck Cancer

Ubiquitination is a post-translational protein modification that is involved in every process of carcinogenesis, such as cell-cycle regulation, proliferation, inhibition of apoptosis, invasion and metastasis and head and neck carcinoma is no exception. Thus the UPS is a candidate target for therapeutic interventions (116) (Table I). The proteasome inhibitor bortezomib is in use for the treatment of multiple myeloma and subtypes of non-Hodgkin lymphoma with impressive therapeutic results, and newer inhibitors such as carfilzomib are in development (117-119). In contrast, in solid tumors, therapeutic results of proteasome inhibition have been modest and bortezomib, although investigated, has not been approved for use in any solid cancer type (120). Specifically in squamous head and neck carcinoma proteasome inhibition has been tested and found to inhibit proliferation of various cell lines and to enhance toxicity of cisplatin, docetaxel and radiation (121-124). A monoclonal antibody against EGFR, cetuximab, has also been found to enhance the inhibitory effects of bortezomib in cell lines (125). Conversely bortezomib enhances the inhibitory effects of another EGFR inhibitor, PKI166, in squamous head and neck carcinoma cell lines by promoting cell death and reducing cell migration through disorganization of the actin filament

Table I. *Examples of therapies for head and neck cancer targeting the UPS. MDM2: mouse double minute 2, βTRCP: β transducin repeat-containing protein, IAP: inhibitor of apoptosis, HPV: human papillomavirus, NF-κB: nuclear factor of κ chains of B cells.*

Therapeutic category	Advantages	Disadvantages
Proteasome inhibitors	In clinical use Development completed	Non-specific, multiple targets Not successful so far in solid tumors Need for identification of sensitive subsets
E1 inhibitors	Possible complete inhibition of tumours highly dependent on them	Non-specific, one target but multiple substrate proteins
MDM2 enzymatic inhibitors	Specific	Expected to be less effective in HPV-related cancer Could target other proteins besides p53
MDM2-p53 interaction inhib.	Specific	Expected to be less effective in HPV-related or *p53* mutant cancer
βTRCP inhibitors	Could be particularly effective in NF-κB-dependent subsets	May produce pro-carcinogenic effects by β-catenin activation for example
IAP inhibitors	Direct inhibitors of core components of apoptosis machinery	Effects on survival of normal cells unknown

network (126). Furthermore, proteasome inhibition by bortezomib enhanced cytotoxicity of histone deacetylase inhibitors trichostatin A and PXD101 both in cell cultures and in xenograft models of mice (127). At least part of the effects of bortezomib were due to inhibition of activated transcription factor NF-κB in this model, given that inhibition of NF-κB by small-RNA interference produced effects similar to bortezomib inhibition in combination with histone deacetylase inhibitors. Additional mechanisms involved in proteasome inhibitor-mediated head and neck cancer suppression in experimental models involve the up-regulation of pro-apoptotic BCL2 interacting modulator (BIM) and BCL2-interacting killer (BIK) proteins and of CDK inhibitor p27 (128, 129). p27 is down-regulated in squamous head and neck carcinoma through ubiquitination with the help of E3 ubiquitin ligase SKP2 and thus, proteasome inhibition promotes its stabilization (129, 130).

Despite the suppressing effects of proteasome inhibition in squamous head and neck carcinoma cell lines and *in vivo* models, there clearly exist differences in proteasome inhibitor sensitivities between various head and neck cell lines (131). These differences have been traced to divergent modulation of transcription factors NF-κB and AP-1. AP-1 subunits such as proto-oncogene jun (c-JUN) are direct proteasome substrates and, as a result, proteasome inhibition increases the activity of this transcription factor contributing to proteasome inhibitor resistance. In contrast, in a bortezomib-sensitive cell line, treatment did not increase AP-1 activity (131). Blockade of c-JUN N-terminal kinase (JNK) that activates AP-1 by the specific inhibitor SP600125 was able to reverse bortezomib resistance in this model (131). Thus, it is evident that the final therapeutic outcome of proteasome inhibition would depend on multiple effects of this inhibition in several parallel and inter-linked pathways. This is an inherent problem of a molecular intervention that acts on a mechanism regulating a myriad of proteins. Determination of baseline and post-inhibition expression of proteins that are known to play crucial roles in head and neck cancer (as discussed in previous sections) and are altered by the UPS and correlation with proteasome inhibitor sensitivity could possibly be a way of predicting sensitivity in order to finally select for patients with head and neck cancer who would benefit from such treatment. In a phase II study of docetaxel combined with bortezomib in metastatic and recurrent head and neck cancer, a DNA microarray analysis showed that patients with progressive disease had higher expression of genes associated with an activated NF-κB pathway than patients that obtained a response or stable disease (132). In another very small study of pre- and post-treatment tumor biopsies, as part of a phase I trial of low-dose bortezomib and re-irradiation of recurrent head and neck cancer, treatment was more effective in reducing activation of REL-A subunit of NF-κB but not other subunits or other activated pathways such as ERK1 and -2, and STAT3, a fact that may explain the lack of significant clinical effect (133).

An alternative strategy would be to intervene at alternative points of the UPS that could offer greater specificity (134, 135). Ubiquitination of p53 by MDM2 could be a target of therapeutic intervention in the minority of head and neck

carcinomas that harbour wild-type *p53* and are not HPV-dependent, given that in HPV cases p53 destruction depends on a different E3 ligase, E6-AP (136). Several inhibitors of MDM2/p53 interaction, such as cis-imidazoline compounds nutlins, as well as thiobenzodiazepine compounds, are under investigation (137, 138). In contrast, use of inhibitors of MDM2 E3 ligase activity could be more complicated given that, as mentioned, MDM2 has other ubiquitination targets such as itself and even EMT inducer SLUG, stabilization of which would have EMT promoting effects (139). This example illustrates the need for in-depth knowledge of the molecular biology for fruitful transfer to the clinical arena. In viral-related head and neck carcinomas, because p53 degradation is facilitated by an alternative E3 ligase, E6-AP, an alternative intervention could involve inhibition of E1 ubiquitin-activating enzyme that serves ubiquitination in a more general manner. Such inhibitors have been discovered and initial studies have shown that they can increase p53 transcriptional activity in cancer cells (140, 141). Nevertheless, problems similar to those encountered by the non-specific actions of proteasome inhibitors could also be a hindrance for the clinical development of E1 inhibitors.

Still other ligases, beyond those targeting p53, may offer therapeutic opportunities and specificity in squamous head and neck carcinoma. Ligase βTRCP which activates NF-κB by ubiquitinating the inhibitor I-κB for proteasomal degradation could be a therapeutic target. An inhibitor of βTRCP has been developed and found to be capable of preventing I-κB degradation (142). A cautionary note is needed here because among other targets of βTRCP is β-catenin which, if stabilized, may act as either a tumor suppressor as part of the adherence junction complex or as a tumor-promoting transcription co-factor. E3 ligase inhibitors of apoptosis (IAPs) could be also a target in head and neck cancer. These RING-type E3s have the advantage that besides taking part in NF-κB pathway activation, they constitute core members of the apoptotic machinery, inhibiting caspase activation (143). Several IAP antagonists are in pre-clinical or initial clinical development (144).

A further therapeutic opportunity may be provided by combinations of ubiquitination-targeting therapies with other targeted interventions. mTOR inhibitors, for example, have been shown to be effective in pre-clinical models of HPV-associated head and neck carcinomas (5) and these could be tested in combination with proteasome inhibitors that would block E6-facilitated degradation of mTOR inhibitor TSC2 (81). In another example, patients with colorectal cancer with *PI3K* mutations have been shown to benefit from aspirin which inhibits cyclo-oxygenase-2 and reduces PI3K activity (145). Aspirin has an additive effect with bortezomib in colorectal cancer *in vitro* (146) and this combination could be also tested in head and neck carcinomas and specifically those bearing *PI3K* mutations.

Development and clinical validation of companion diagnostics would greatly facilitate the clinical progress of any targeted intervention as it is unlikely that a particular targeted therapy will be effective for all patients with head and neck cancer, despite their categorization into defined pathophysiological causative subtypes. Such predictive markers recapitulate the very essence of targeted treatments and may be in fact a prerequisite for their successful development.

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