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The Role of the NF-kappaB Transcriptome and Proteome as Biomarkers in Human Head and Neck Squamous Cell Carcinomas*

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Summary

NF-κB is a family of signal activated transcription factors comprised of hetero- or homo-dimers from 5 different subunits, NF-κB1, NF-κB2, RELA, cREL and RELB. NF-κBs normally are transiently activated in response to infection or injury, but in cancers are aberrantly activated, regulating a transcriptome of hundreds of genes and corresponding proteome that promote pathogenesis and therapeutic resistance. In head and neck squamous cell carcinomas, an important role of NF-κB in regulation of the altered transcriptome and proteome has been established, providing a catalog of activating and target genes and proteins that may be useful as biomarkers of alterations in this pathway for this and other cancers. An emerging appreciation that NF-κB and other signal pathways form an altered regulatory network highlights the need to use biomarkers and combine targeted agents for personalized therapy of cancer.

Keywords

NF-κB; proteome; transcriptome; signal pathways; head and neck cancer

Introduction

NF-κB is an evolutionarily conserved family of signal activated transcription factors comprised of hetero- or homodimers from 5 different subunits, NF-κB1, NF-κB2, RELA, cREL and RELB [1]. NF-κB1 is translated as p105 and processed to p50, and NF-κB2 is translated as p100 and processed to p52 following phosphorylation and proteasome-dependent degradation of their ankyrin repeat-containing c-termini. RELA (p65), cREL and RELB are synthesized in mature form and often form heterodimers with NF-κB1 or NF-κB2. NF-κB1 is often associated with RELA (or cREL) and bound in the cytoplasm in an inactive form by Inhibitor-kappaB (IκB)-α, β or γ, and known as the canonical, or classical form of NF-κB (Figure 1A). This form is activated by various components of pathogens or cytokines such as Tumor Necrosis Factor (TNF) or Interleukin-1 (IL-1), primarily via a trimeric Inhibitor-kappaB Kinase (IKK) comprised of α, β and γ subunits, that phosphorylates IκB, leading to IκB proteasomal

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degradation, and nuclear translocation and DNA binding of the NF- κ B1/RELA heterodimer. It regulates genes that function to mediate cytoprotective responses as well as innate and adaptive immune responses. NF- κ B2 often forms a heterodimer with RELB, known as the non-canonical form of NF- κ B. The non-canonical or alternate pathway is activated by other stimuli such as lymphotoxin B, BAFF and CD40L, via an NF- κ B Inducing Kinase (NIK) and IKK α dimeric complex (Figure 1B). Studies in transgenic mice suggested the non-canonical pathway has a more restricted role in B lymphocyte development and immunity.

Cumulative evidence indicates that NF- κ B is aberrantly activated in the majority of cancers, and plays a role in the promoting carcinogenesis and malignant progression [2–4]. Aberrant activation of the canonical pathway has been broadly implicated in development of many cancers, consistent with its ubiquitous expression and role in promoting cell survival and growth. Aberrant activation of the non-canonical pathway has been demonstrated in B lymphoid malignancies, as well as other cancers.

1. Aberrant activation of NF- κ B in head and neck cancer

a. Aberrantly activated NF- κ B family members and related regulatory pathways

Human head and neck squamous cell carcinomas (HNSCC) were among the first cancers for which evidence for aberrant constitutive activation of NF- κ B was obtained, using cell lines derived from patient tumors [5]. Electromobility shift assays demonstrated increased nuclear DNA binding of the classical heterodimer NF- κ B1/RELA (p50/p65), and to a lesser extent, cREL and non-canonical NF- κ B2 containing complexes [6]. Nuclear localization of RELA was confirmed in HNSCC tumor tissue, and inhibition of this nuclear activation in tumor xenografts by forced expression of a phosphorylated mutant of I κ B was demonstrated to inhibit tumorigenesis, indicating NF- κ B plays a key role in the malignant phenotype of HNSCC [7]. Subsequent studies have shown that NF- κ B is activated in squamous dysplasias and carcinomas, with the intensity of nuclear immunostaining serving as a proteomic marker correlated with progression of dysplasia and decreased survival in patients with HNSCC [8]. Aberrant NF- κ B activation has been detected in tobacco-associated as well as viral-related HNSCC, such as EBV-related nasopharyngeal and HPV associated oropharyngeal carcinomas [5]. Evidence for a role for both IKK α and IKK β in NF- κ B activation [9] and nuclear activation of all five NF- κ B subunits has recently been demonstrated [10], consistent with activation of both canonical and non-canonical pathways and NF- κ B components in HNSCC (Figure 1).

b. Elevated proinflammatory and proangiogenic cytokines, and other NF- κ B target molecules in cell lines, serum and tissue specimens of head and neck cancer patients

A prominent portion of the classical NF- κ B target gene program includes chemokines and cytokines, which promote inflammation and angiogenesis (Figure 1). In addition to the role of NF- κ B to directly promote cancer cell survival, these factors provide selective advantages for tumor growth in the microenvironment via stimulation of inflammatory cell infiltration and angiogenesis [2]. Consistent with the aberrant activation of NF- κ B in the majority of HNSCC, many cell lines and tumor specimens were found to over-express a repertoire of chemokines/cytokines *in vitro* and *in situ*, that include IL-1 α , Interleukin 6 (IL-6), Interleukin 8 (IL-8), granulocyte-macrophage colony-stimulating factor (GM-CSF), growth-regulated oncogene-1 (GRO-1), and vascular endothelial growth factor (VEGF, Figure 2) [11–16]. In response to some of these tumor-derived factors, tumor fibroblasts have been shown to produce IL-6 and scatter factor (SF)/hepatocyte growth factor (HGF) [17]. IL-6, IL-8, GRO-1, VEGF, and HGF are of sufficient stability and are produced at high enough concentrations that increased levels have been detected in the serum of patients when compared to controls without cancer, or with benign laryngeal squamous papillomatosis [12,18]. Decreasing cytokine levels were associated

with better responses to therapies, while increasing levels were related to progression or recurrence, and decreased survival [15,19].

IL-1 is a cytokine that initiates proinflammatory responses through regulating NF- κ B activation and triggering cascade expression of chemokines/cytokines detected in SCC [14, 17,20,21]. IL-1 can serve as an autocrine factor to stimulate HNSCC to produce IL-6, IL-8, GM-CSF, and VEGF, as well as a paracrine factor to stimulate production of HGF by stromal fibroblasts [14,18,20,21, Chen Z, unpublished observations]. In addition, IL-1 has important systemic regulatory effects as a mediator of acute-phase reactions, increased catabolic state and cachexia often observed in patients with aggressive HNSCC [12]. IL-1 and IL-6 both show direct effects as autocrine or paracrine factors that stimulate proliferation of HNSCC cells [14,21,22]. Through DNA microarray and genome-wide bioinformatics analysis, we identified the dysregulation of IL-1 pathway in HNSCC as due to defective expression of the inhibitory molecules of IL-1, including IL-1R2, a decoy receptor which only binds to IL-1 ligand without transducing the downstream signals, and IL-1R antagonist (IL-1RN), which is able to competitively bind to IL-1R to block the signals [23–25, Bagain et al, manuscript in preparation]. We identified a significantly decreased expression of mRNA and protein levels of IL-1R2 and IL-1RN in HNSCC cell lines when compared with normal cells [23–25, Bagain et al, manuscript in preparation]. This supports the hypothesis that overactivation of the IL-1 pathway is due to the deficiencies in the negative regulators, which could be one of the upstream signals contributing to the aberrant activation of NF- κ B in HNSCC.

The downstream targets of IL-1 and NF- κ B pathways, such as IL-8 and GRO-1, are members of a related family of chemoattractant and proliferative factors that contain a cysteine-X-cysteine (C-X-C) amino acid motif. IL-8 and GRO-1 have been shown to serve as chemoattractants for neutrophils, monocytes and endothelial cells, which are major constituents of the inflammatory response in HNSCC [12,14,26]. Genome-wide analysis of gene expression profiles of both human HNSCC lines and a murine metastatic SCC model consistently identified and linked IL-8/GRO-1 over-expression to the phenotypes of more aggressive growth and metastases, angiogenesis, and inflammatory cell infiltration in squamous cell carcinoma [23,27]. The direct regulation of IL-8/GRO-1 expression by NF- κ B was confirmed with over-expression of mutant I κ B α , an inhibitor of NF- κ B activation, in both human HNSCC cell lines and a murine SCC metastatic model, where the aggressive pattern of tumor growth was inhibited, also [7,28]. In addition, the aggressive SCC growth is reversed in knockout mice deficient in CXC receptor 2, the receptor for the GRO-1 chemokine [13]. Kitadai et al. have shown that IL-8 has similar effects on growth of other histologic types of human tumors as xenografts in mice [29]. These results provide direct evidence that these tumor factors and mediated host responses are critical in tumor progression and metastasis of SCC.

Other proinflammatory and proangiogenic cytokines and growth factors, such IL-6, VEGF and HGF were also identified at elevated levels in the supernatants of HNSCC cell lines and patient serum [12]. IL-6 gene expression has been shown to be directly regulated by NF- κ B [30], and IL-6 protein directly binds to IL-6R to mediate JAK/STAT3 activation and promote tumor cell survival and resistance to chemo- and epidermal growth factor receptor (EGFR) targeted therapies [30, Pernas et al, manuscript submitted]. The expression of VEGF and HGF may be indirectly modulated by NF- κ B activation. Proteasome inhibition of NF- κ B activation by bortezomib significantly suppressed the expression of IL-8 and VEGF in HNSCC cell lines, as well in patient serum [31]. Over-expression of the NF- κ B inhibitor I κ B α suppressed VEGF expression in a murine SCC model [20]. VEGF expression is modulated by pharmacologic inhibitors of pathways upstream of NF- κ B and AP-1 [11,32,33]. While the relationship between NF- κ B and HGF remains unclear, preliminary evidence suggests that NF- κ B-induced expression of IL-1, IL-6, and IL-8 may stimulate HGF production in stromal fibroblasts, therefore indirectly linking aberrant NF- κ B activation in HNSCC with elevated serum HGF

(Chen Z, unpublished observations). Conversely, HGF is able to induce IL-8 and VEGF production by HNSCC cells in cultures [18], which form a positive paracrine regulatory loop to further promote tumor growth and angiogenesis (Chen Z, unpublished observations). Results from our clinical trials support our laboratory findings, that serum IL-6, IL-8/GRO-1, VEGF, and HGF levels were elevated and changed consistently with tumor response, relapse, or patient complications, and that these changes are associated with patient survival [12,15,19,34]. Experimental and clinical evidence supports functional contribution of these factors to the phenotype and pathogenicity of SCC and other cancers, and therefore serves to support use of these factors as candidate serum biomarkers [11–27].

c. Unique gene signature and transcriptome under the regulation of NF- κ B pathways as biomarkers for subsets of head and neck cancer

Gene expression profiles have been intensively studied to identify the critical gene expression signatures related to heterogeneity in cancer phenotypes, which accelerate and broaden our understanding of the malignant process involved in multiple genetic and biological defects [35–37]. However, relatively limited information has been generated from large-scale analyses of gene expression profiles in cancer related investigations to study the transcriptional control of global gene expression. To address this question, we globally analyzed the gene expression profile of ten HNSCC cell lines and normal keratinocytes, and conducted a bioinformatic analysis of the promoters and signal network relationships of the genes differentially expressed by HNSCC [23–25]. Among the gene signatures differentially expressed by HNSCC lines, we identified several gene clusters with dominant NF- κ B binding sites [23–25]. In this study, a specific gene cluster (cluster B) was identified with NF- κ B binding motifs in ~70% genes (Figure 3). This signature includes many genes involved in the early response and injury, such as *BIRC2* (baculoviral IAP repeat-containing 2), *CROC4* (transcriptional activator of the c-fos promoter), *DMAP1* (DNA methyltransferase 1-associated protein 1), *ICAM1* (intercellular adhesion molecule 1, CD54), *IL-6*, *IL-8*, *PORIMIN* (pro-oncogenic receptor inducing membrane injury gene), *RAB17* (RAB17, member Ras oncogene family), *STK6* (serine/threonine kinase 15), *YAP1* (Yes-associated protein 1) [23–25]. In addition, NF- κ B regulation of these genes was experimentally validated by TNF- α treatment in HNSCC cell lines, and among the genes significantly induced were known NF- κ B target genes, such as *IL-1 α* , *IL-1 β* , *IL-6*, *IL-8*, *GM-CSF*, *ICAM1*, and *TNFAIP2*, and other newly identified and less studied genes containing NF- κ B regulatory sites in their promoter [23–25]. We also identified a large program of NF- κ B related genes by promoter and ontology analysis of genes differentially expressed with metastatic progression in a murine SCC model, and confirmed that the expression of many of these genes and the malignant phenotype were restored to that of normal keratinocytes by inhibition of NF- κ B by mutant I κ B α [27,28]. Consistent with our analyses, Chung et al have recently reported that human homologues of a set of 99 of the genes in this murine study were highly represented in a gene cluster and subset of patients with HNSCC at high risk for recurrence and decreased survival [38].

Utilizing array technology together with state-of-the-art bioinformatic analyses, we identified five transcription factors with increased prevalence and differential distribution associated with differentially expressed gene signatures in subsets of HNSCC cell lines [23,24]. NF- κ B is one of them, and together with p53, AP-1, STAT3 and EGR-1 transcription factors, have all been previously implicated as independent factors contributing to malignant progression of HNSCC [6,7,11,33,37,39–41]. Our recent study revealed that regulatory sites for transcription factors NF- κ B, p53 and AP-1 are more prevalent in promoters that control gene signatures over-expressed in subsets of HNSCC, whereas, STAT3 and EGR1 sites were more broadly distributed among the repertoire of genes over-expressed by the full panel of HNSCC studied [23–25] (Figure 4). Furthermore, using a newly developed mathematical modeling program, COGRIM, we predicted 748 putative NF- κ B target genes, which consisted of 59% of 1265

differentially expressed genes from microarray in HNSCC cell lines [25]. This percentage is higher than that of all predicted NF- κ B binding motifs calculated in vertebrates, and is remarkably consistent with the approximate percentage of genes modulated by an inducible mutant I κ B α in expression profiling studies in our murine SCC model [28]. Together, these experimental and *in silico* analyses of expression profiling data in human HNSCC and murine SCC are consistent with the conclusion that NF- κ B is involved as a key regulatory factor in global alterations of gene expression.

As the NF- κ B family consists of five subunits, and different hetero- or homodimers exhibit differential regulatory activities [1], the direct interactions or close regulation by various NF- κ B subunits of these target genes were analyzed by Genomatix and Ingenuity software. Many known NF- κ B target genes, such as *CCDN1*, *CSF1*, *CSF2*, *ELF3*, *ICAM1*, *IL-1A*, *IL-1B*, *IL-1RN*, *IL-2RA*, *IL-6*, *IL-8*, contained sites with features consistent with regulation by RELA [25]. In addition, the up-regulated NF- κ B target genes were enriched for components of important signal pathways and functions implicated in all of the HNSCC cell lines studied (Table 1), including signaling and functions related to ephrin receptor, IL-6 signaling, p38 and ERK/MAPK, inositol phosphate metabolism, leukocyte extravasation signaling, and xenobiotic metabolism pathways [25]. The pathways and target genes identified are consistent with previous evidence from studies by us and others that NF- κ B promotes proinflammation, pro-angiogenesis, cell adhesion and migration through up-regulation of some of these genes [2]. The inositol phosphate metabolism pathway consists of molecular components of PI3K and PKC pathways, which have both important signal pathways implicated in promoting tumorigenesis, especially in epidermis and epithelia [42–44]. The involvement of NF- κ B in the down-regulated genes were identified in Wnt/ β -catenin and TGF- β pathways, including many negative regulators of cell growth and survival in tumorigenesis in epidermis and epithelia [45]. Deficiency of *TGF β R2* has been demonstrated in HNSCC cell lines and tissue specimens [46, Cohen J, manuscript in preparation]. Other signal pathways identified are more specific to the subsets of HNSCC cells with phenotypic and genotypic differences related to p53 status. For cells with wild type (wt) p53-deficient status, altered gene expression included genes involved in cell cycle/G2/M checkpoint, Neuregulin signaling, PPAR signaling and Ubiquitination pathways [25]. The pathways related to growth factor (insulin growth factor, IGF), integrins, intermediate signals (NF- κ B and SAPK/JNK), cytokines (VEGF and GM-CSF) are dominant in cells with mutant p53 status [25].

Our genomic and transcriptomic study provided a strong link between NF- κ B and related pathways identified by systems biology approaches, supporting an hypothesis that the malignant progression of HNSCC is due to, and/or leads to, multiple genetic and phenotypic defects, such as p53 mutation or underexpression [40,47], and activation of several major growth factor and cytokine receptor pathways, including TNFR [48], IL-1R [14,21], IL-6R [30], EGFR [33], HGFR/cMet [18], and PDGFR [48], as well as defective expression affecting others, such as TGF β RII [46, Cohen J, manuscript in preparation] that are linked to aberrant NF- κ B signaling and transcriptional control. These receptors modulate multiple signal pathways, including aberrant activation of NF- κ B [6–8], AP-1 [6,21,49], JAK/STAT [30], EGR1 [41], CK2 [9], MAPK [10,11,18], PI3K [18,33], SMADs [46, Cohen J, manuscript in preparation] and BCL-XL/IAP associated apoptosis pathways [50,51], consistent with function of NF- κ B as part of a more complex signal network. All of these pathways have been significantly and independently implicated in HNSCC tumorigenesis.

3. NF- κ B regulated proteomes and transcriptomes as biomarkers and therapeutic targets in preclinical and clinical studies of head and neck cancer

a. NF- κ B regulated proteome and transcriptome in murine syngeneic SCC models

We previously developed a syngeneic murine model that includes the spontaneously transformed BALB/c keratinocyte line Pam 212, and rare lymph node and lung metastases (LY and LU) of Pam 212 [16,52]. The metastatic Pam LY and Pam LU cell lines were found to form tumors and metastases at a higher rate than the parental Pam 212 tumor line *in vivo* [16, 52]. The aggressive phenotypes are related to an increased expression of a repertoire of proinflammatory and proangiogenic factors that are regulated by transcription factor NF- κ B [27,53]. Utilizing murine cDNA microarrays to compare the stepwise changes accompanied with tumor progression and metastasis, we identified a group of up-regulated genes, with the functionality involved in immunologic, inflammatory and angiogenesis responses, such as *Gro-1*(KC), *C3* (complement component 3), *Il-12b*, *Csf-1* and *Spp1* (Osteopontin); signal transduction and regulation of gene expression and DNA replication, such as *c-Met*, *Ntrk2* (neurotrophic receptor tyrosine kinase), *HMG-1(Y)*, and replication protein A (14 kd subunit); and modulation of cell cycle and apoptosis, such as *cIAP(Birc2)*, *FasL* (Fas ligand), *PEA-15*, and ubiquitin-activating enzyme E1 [27]. Bioinformatic analysis revealed that many of the genes are targets or involved in regulation by NF- κ B signal transduction pathway. Specifically, inhibition of NF- κ B decreased *Gro-1* expression in LY-2 cells, which is associated with decreased angiogenesis and tumorigenesis [13,27]. We showed that enforced expression of *Gro-1* in low-*Gro-1* expressing Pam 212 cells converted these cells to a metastatic phenotype and promoted increased angiogenesis, tumorigenesis, and metastasis [13,20].

In a subsequent study, we profiled the gene expression after conditionally inhibiting NF- κ B by inducible I κ B α mutant (I κ B α M) in the metastatic Pam LY-2 cells using cDNA microarray [28]. We identified a cluster of 308 genes differentially expressed, with 141 genes decreased and 167 genes increased, in association with transformation, tumor progression, and activation of NF- κ B [28]. Remarkably, expression of I κ B α M in Pam LY-2 reversed the gene expression patterns accompanied with tumor progression (<http://www.nidcd.nih.gov/research/scientists/vanwaesc.asp>). The genes were also classified according to putative functions and published associations with NF- κ B as determined by bioinformatic analyses, and it was confirmed that many genes detected as upregulated in LY2 cells had been associated with NF- κ B. Furthermore, the results of this study confirmed that over-expression of I κ B α M inhibits NF- κ B activation, which suppresses the protein expression of cyclin D1 (*Ccnd1*), *cIAP* and *Gro-1*, and reduces the expression of β -catenin in SCC. These molecules have been implicated with critical functions in tumorigenesis. Cyclin D1 is overexpressed in the majority of HNSCC and promotes proliferation and tumorigenesis [50]. Increased expression of *cIAP* was previously detected in metastatic SCC and shown to promote survival in many cancers [23,27,28]. *Gro-1*, one of the genes consistently detected in LY-2 and aggressive SCC, was also previously shown to be modulated by NF- κ B and to contribute to angiogenesis, tumorigenesis, and metastasis [13,20,27,53]. Decreased expression of β -catenin and related genes has been observed in HNSCC cell lines [25] and is associated with metastasis of human oral SCC [54].

b. NF- κ B biomarkers in testing of newly developed therapeutic agents in preclinical *in vitro* and *in vivo* animal models

I. Proteasome Inhibitor (Bortezomib/VELCADE™/PS-341)—Bortezomib has been developed in recent years for molecular targeting and inhibition of the proteasome, a complex which mediates the turnover of many intracellular proteins, including those controlling cell signaling, survival, and cell cycle regulation [55,56]. Bortezomib selectively inhibits proteasome activity, which is required for activation of NF- κ B and degradation of multiple

components of signal pathways involved in the pathogenesis of cancer [55,56]. Bortezomib can inhibit the NF- κ B pathway through its inhibitory effects on degradation of ubiquitinated I κ B, which binds and sequesters NF- κ B in the cytoplasm, inhibiting its nuclear localization and binding to the promoters of target genes [55–59]. The inhibitory activity of bortezomib has been demonstrated against a spectrum of cancer cells in culture [57–67] and in animal models [50,68–70] including suppression of NF- κ B and other signal transcription pathways with induction of cell apoptosis and cell cycle arrest. The molecular and clinical effects of bortezomib and potential mechanisms of sensitivity and resistance have been most extensively studied in multiple myeloma (MM) and certain other hematopoietic malignancies [58,60–63], and to a lesser extent in solid cancers [31,50,57,64–68,70,71].

We examined the effects of bortezomib on activation of NF- κ B, cell survival, growth, and angiogenesis in murine and human SCC cell lines, as well as in tumor models *in vivo* [49,50,71]. Bortezomib inhibited activation of NF- κ B DNA binding and functional reporter activity in the nanomolar concentration range. Bortezomib induced cytotoxicity was observed in four murine and two human SCC lines, and followed early cleavage of PARP, a marker of caspase-mediated apoptosis. *In vivo*, bortezomib inhibited growth of murine and human SCC in mice, which was associated with a marked decrease in vessel density. In addition, bortezomib inhibited expression of proinflammatory and proangiogenic factors GRO-1 and VEGF by SCC in the range at which bortezomib inhibits NF- κ B [71].

In a phase I clinical trial of bortezomib with re-irradiation for patients with recurrent HNSCC, we observed heterogeneous responses, with 5/17 patients demonstrating tumor shrinkage >30%, meeting RECIST criteria for partial response, and 3/4 with evaluable pre and post treatment biopsies demonstrating inhibition of phospho-p65 staining in association with increased apoptosis by TUNEL assay [10,31]. These observations lead us to study the role of NF- κ B and other potential mechanisms in the differential sensitivity to bortezomib in a panel of HNSCC lines. We observed that the differential sensitivity to bortezomib among the HNSCC cell lines was not strictly proportional to the baseline differences in activation of NF- κ B, indicating that other NF- κ B-independent mechanism(s) may be involved [58,60–67]. Comparison of two cell lines UM-SCC-11A and –11B, which showed similarly high levels of NF- κ B activation, revealed differences in the kinetics and extent of proteasome inhibition, ubiquitination, and AP-1 activation as underlying the difference in sensitivity [49]. We showed that bortezomib inhibited proteasome and NF- κ B activities in UM-SCC-11A cells to a lesser extent when compared with –11B cells, and increased AP-1 activity in UM-SCC-11A cells was observed after treatment with bortezomib. Blockage of upstream signal kinases JNK and other MAPKs could reduce AP-1 activity and synergize with bortezomib anti-tumor activity in UM-SCC-11A cells. JNK and AP-1 activation via histone deacetylase-6 by bortezomib has recently been implicated in protective autophagy pathway responses in other cancers [72]. Our data suggest that the mechanisms underlying these differences may exist at various levels of regulation, from differences in the extent of proteasome inhibition through variable effects on NF- κ B and AP-1 transcription factor activity. This pre-clinical study suggested that assays comparing the effects of bortezomib in pre- and post-treatment biopsies on proteasome activity, NF- κ B and AP-1 signal activation, proliferation, and apoptosis could be useful to develop biomarkers for clinical trials of cancers, in which heterogeneous responses to bortezomib have been observed. We have recently demonstrated relative specificity of effects of Bortezomib on NF- κ B relative to ERK and JNK, which activate AP-1, STAT3, and AKT, in pilot studies using pre and post treatment biopsies [10]. We previously showed that NF- κ B regulated expression of genes *CCND1*, *BCL-XL*, *IL-6* and *VEGF*, and serum proteomic markers IL-6 and VEGF were down-modulated in patients receiving Bortezomib [31]. Together, the phospho-signaling biomarkers could be relevant for selecting regimens to combine, and for determining molecular response to agents targeting NF- κ B and these other pathways such as JNK for more effective

therapy in HNSCC. The specificity and sensitivity of these assays will need to be determined in larger trials.

II Histone Deacetylase Inhibitor (PXD 101/Belinostat)—The multiple transcriptional, genetic and biological alterations involved in HNSCC prompted us to investigate a therapy targeting NF- κ B with therapeutic agents more broadly targeting transcription, in an effort to develop a more effective combination therapy. The proteasome inhibitor, bortezomib, was studied in combination with classic histone deacetylase (HDAC) inhibitors (HDIs), and newer agents in clinical trials, such as PXD 101. HDIs represent an important class of agents with anticancer activity, through induced accumulation of acetylated histones and other proteins regulating chromatin structure and transcription, thereby altering the transactivation and expression of specific genes that regulate cell growth arrest, differentiation, or apoptosis [73–76]. The newer synthetic HDIs with more favorable pharmacologic characteristics for therapeutic investigation include phenylbutyrate, depsipeptide, SAHA, MS275, and PXD101 [74–76]. In preclinical studies, HDIs have shown variable antiproliferative and cytotoxic activity in a wide range of solid malignancies, including head and neck, breast, colon, lung, and ovarian cancers, as well as hematologic malignancies, such as lymphomas, leukemias, and myeloma [74–76]. In HNSCC, classic HDIs have shown modest antiproliferative and cytotoxic activity when used alone [77,78] and greater activity when used in combination with radiation and demethylating agents [79,80].

Regulation of NF- κ B1/RELA DNA binding, transactivation, and turnover has been shown to involve acetylation of RELA by histone acetylase CBP/p300 and deacetylation by HDACs [81,82]. Given the role of NF- κ B1/RELA in prosurvival mechanisms in HNSCC, we hypothesized that the relative resistance of HNSCC to HDIs could result from the enhanced activation and acetylation of NF- κ B, and expression of NF- κ B regulated genes known to promote cell survival [81,82]. Our study showed that the classic HDIs TSA and NaBu enhanced basal transactivation, DNA binding and acetylation of RELA, and expression of NF- κ B regulated proliferative and antiapoptotic genes [50]. In addition, specific NF- κ B inhibition by RELA siRNA blocked HDIs induced activation of NF- κ B activity and target gene expression, such as *CDKN1A* (*p21*), *CCND1* (cyclin D1), and *BCL-XL* (BCL2-like 1), induced cell cycle arrest and cell death, and sensitized HNSCC to HDIs. We further examined the cytotoxic effects of combining a novel HDI, PXD101, with proteasome inhibitor bortezomib *in vitro* and in mice bearing human HNSCC xenografts *in vivo*. Bortezomib increased cell sensitivity to HDIs, including PXD101 *in vitro*, and promoted the antitumor effects of PXD101 in bortezomib-resistant UM-SCC-11A xenografts. However, gastrointestinal toxicity, weight loss, and mortality of the combination were dose limiting and could be alleviated with parenteral fluid administration. We conclude that HDI-enhanced NF- κ B activation is one of the major mechanisms of resistance of HNSCC to HDIs. The combination of HDI and proteasome inhibitor produced increased antitumor activity. However, low starting dosages and short exposures for clinical studies combining HDIs with proteasome inhibitors and IV fluid support may be warranted for future clinical trials [50].

III. Detection of molecular responses to EGFR inhibitor Gefitinib by Immunohistochemistry, Reverse Phase Protein Microarray and Western blot—

Increased understanding of molecular alterations in HNSCC and other cancers have ushered in efforts to develop other molecular-targeted therapies of cell signaling. Among these alterations, over-expression of EGFR has been identified in many cancers, including 80–100% of HNSCC [83–85], where it has also been implicated in a more aggressive phenotype, increased resistance to treatment, and poorer clinical outcome [86,87]. We showed that EGFR over-expression and signaling activate NF- κ B pathways and downstream genes [33], and the crosstalk between NF- κ B and other classical down-stream pathways of EGFR, such as AKT, MEK and STAT3 to promote gene expression, has been demonstrated in these HNSCC cell

lines [25] (Figure 2). These observations have led to interest in the study of molecularly targeted small molecule inhibitors of EGFR for the treatment of HNSCC. Gefitinib (Iressa®), an EGFR tyrosine kinase inhibitor [88–90], has been tested in clinical trials in solid tumors, including HNSCC, as the single agent, or in the combination with other chemotherapies or radiation, but has shown limited clinical efficacy as a single agent with response rates of 10–15% [91–95]. Molecular biomarkers that could serve as surrogates to identify patients responsive to gefitinib or other EGFR inhibitors would be useful in selection of patients for therapy.

Using a panel of four UM-SCC cell lines, we examined EGFR and downstream molecular pathways potentially activated by EGFR or other mechanisms in HNSCC that could serve as molecular surrogates of sensitivity or resistance to EGFR inhibitors. The four cell lines exhibited different levels of total EGFR and EGF-induced receptor phosphorylation, and UM-SCC-11A and -11B cell lines exhibited higher levels total EGFR phosphorylation, which are consistent with our and other reports of over-expression EGFR in HNSCC [83–87,96]. However, drug sensitivity of the cell lines could not be simply explained by the level of total EGFR, or differences in basal nor EGF-induced EGFR phosphorylation, in that gefitinib induced strong suppression of all EGFR phosphorylation sites in all of the cell lines tested.

The gefitinib sensitivity in HNSCC lines appeared to correlate most closely with the extent of phosphorylation of the individual EGFR sites and their target molecules, which include AKT, ERK and STAT3 signal molecules. Phosphorylation at EGFR Tyr845 of the kinase domain is involved in c-SRC activation and regulated by both EGF-and integrin-mediated EGFR activation [86,87,97]. Tyr1068 serves as docking site for PIP3K and growth factor binding protein 2 (Grb2), and these molecules mediate activation of AKT and ERK pathways [93,98–100]. The pair of phosphorylated residues, Tyr1148 and Tyr1173, provides a docking site for the Shc scaffold protein, and both sites are involved in MAP kinase signaling activation [99, 100]. In addition, Tyr1173 is the favored autophosphorylation site in ligand-activated wild-type EGFR and appears to be the major site of autophosphorylation in the mutant EGFRvIII [95,101]. The drug sensitivity (IC₅₀) in these HNSCC cell lines was negatively correlated with levels of basal and EGF induced pAKT, and positively correlated with EGF induced pSTAT3. ERK1/2 and NF-κB signals also contributed to survival mechanisms responding to gefitinib. Our data are consistent with previous preclinical studies in lung cancer cells that harbor EGFR mutations [102–107], where the drug sensitivity to gefitinib is closely correlated with EGFR-dependent AKT and ERK activation.

In addition, we cross-validated the phospho-EGFR, NF-κB, ERK, AKT and STAT3 immunohistochemistry biomarker responses identified using several methods, including Western blot analysis, ELISA and reverse-phase protein microarray (RPMA), a newly developed proteomic platform, which is capable of measuring numerous specific phosphorylated proteins that are involved in important signaling pathways from a small tissue biopsy or a few thousand cells [108,109]. The advantages of this technology are the small sample size required, as well as the quantitative and sensitive protein measurements permitted with use of high quality validated antibodies. A 1 cm long core biopsy can yield up to 100 RPMA slides, which makes it possible to screen a panel of biomarkers [108–110], in contrast to the extensive usage of tissue specimen by classical immunohistochemistry staining or other testing methods. Secondly, this method is able to generate the quantitative measurement of the specific biomarkers using a fitting curve with serial dilution of the samples, whereas classical immunohistochemistry is only semi-quantitative. The successful cross validation of biomarkers identified *in vitro*, demonstrates the feasibility of applying the newly developed RPMA technologies in clinical trials where a major challenge is the limited quantity of clinical samples from patients. In this study, we obtained data consistent with the results using classical immunohistochemistry (Van Waes C, *et al*, manuscript submitted) and the studies from UM-SCC cell lines (Pernas FG C, *et al*, manuscript submitted). We observed that the highest

baseline pretreatment level of pAKT473 (an activator of NF- κ B and mTOR pathways), and post-gefitinib suppression of pEGFR1148, pAKT473, pERK185/187, and pNF- κ B p65-ser536 occurred in a molecular and clinical responder (Pernas FG C, *et al*, manuscript submitted). In addition, an increase of cleaved caspase 3 activation was also observed in the responder, which is consistent with the tumor apoptosis detected by TUNEL assay (Van Waes C, *et al*, manuscript in preparation). Together, these findings indicate that EGFR-dependent co-activation of these multiple pathways and gefitinib sensitivity occurs infrequently in a subset of patients with HNSCC. In contrast, gefitinib failed to inhibit or even enhanced phosphorylation of MEK, STAT3 and/or NF- κ B pathway components, and had no effect on caspase activation in the rest of non-responder patients. This study again revealed the clinical and molecular heterogeneity of cancer as a major hurdle that limits effective treatment. Such heterogeneity usually remains undetected by standard histologic pathological classification and clinical grading systems. Our study suggests that by utilizing IHC and /or RPMA, it may be feasible to determine which pathways are activated and how they correlate with prognosis, select therapy targeting these pathways, and to use changes in phosphorylation, expression of protein and functional changes such as TUNEL or caspase cleavage together as early biomarkers of response to the treatment.

c. Clinical studies of newly developed therapeutic agents in head and neck cancer patients

We have recently conducted unblinded dose escalation phase I clinical trials combining the proteasome inhibitor bortezomib, and the EGFR inhibitor gefitinib with radiation therapy for patients with HNSCC, with an initial phase of treatment with the drugs alone to study the effects on transcriptional and proteomic markers. EGFR may promote co-activation of the AKT-NF- κ B, MAPK and STAT3 pathways [23,33], and gefitinib and bortezomib have potential to inhibit NF- κ B activation at different levels (Figure 4).

Bortezomib, 0.6mg/m², was shown to inhibit NF- κ B nuclear localization by immunostaining in 3/4 subjects and expression of NF- κ B target genes important in pathogenicity of HNSCC by quantitative RT-PCR analysis was inhibited in 2/2 specimens, when pre- and 24-hour post-treatment biopsies were compared [31]. Inhibition of NF- κ B, Cyclin D1 and BCL-XL were consistent with reductions in Ki-67 and increased TUNEL staining observed. As only partial responses to the combination of bortezomib and re-irradiation in the study were observed, we investigated if non-canonical NF- κ B and other signal pathways such as MAPK and STAT3 were activated and differentially affected by proteasome inhibitor treatment [10]. In addition to NF- κ B1 and RELA, cREL and non-canonical subunits NF- κ B2 and RELB demonstrated nuclear localization, and were not significantly inhibited in most of the tumors studied. Further, bortezomib did not inhibit ERK1/2, JNK and AP-1, AKT or STAT3 nuclear activation. In some tumor specimens, increased ERK or STAT3 staining was perceptible, consistent with potential induction of AP-1 or STAT3. These observations suggest that the non-canonical NF- κ B and other co-activated signal pathways may provide other potential mechanisms supporting remaining expression of pro-survival genes and tumor resistance.

The effects of gefitinib were studied after one week of administration of 250mg daily before treatment with weekly paclitaxel and radiation in patients with Stage III/IV HNSCC (Van Waes, manuscript in preparation). Tumor specimens from only 1/7 subjects showed inhibition of phosphorylation of EGFR and downstream signal proteins ERK1/2, AKT, NF- κ B and STAT3 together with anti-proliferative and apoptotic responses and measured by inhibition of Ki-67 and increased TUNEL staining. As described above, these findings were cross validated and independently confirmed using reverse phase protein microarray analysis (Pernas, FG, manuscript submitted). This patient was one of the 5/10 who had a complete response, where presumably the remaining 4 showing lack of response to gefitinib were due primarily to the standard cytotoxic chemo and radiation therapy. These results are consistent with a dominant role of EGFR in co-activation of these pathways in only a small subset of patients, and

sensitivity of only 10–15% of clinical responders observed in larger trials. Although these are pilot studies, these phospho-protein analyses indicate that important oncogenic pathways are coactivated in the majority of HNSCC, with or independent of EGFR phosphorylation. Together with Ki-67 and TUNEL marker responses, the limited co-inhibition of pathways downstream by EGFR inhibitor are consistent with the low clinical response rates in single agent trials, and may therefore warrant study as early markers of sensitivity of molecular signaling and cytotoxic response to EGFR inhibitors in the limited subset of patients in which signaling and cell survival are dependent on EGFR.

Conclusions and Future Perspective

Characterization of the NF- κ B transcriptome and proteome has led to the demonstration of frequent NF- κ B nuclear localization and target molecule expression as biomarkers associated with pathogenicity and survival of HNSCC, while inhibition or decrease in these markers is observed with agents that inhibit NF- κ B or induce tumor responses, making them potentially useful in selection and monitoring of response to therapy. Evaluation of the role of NF- κ B in the wider context of alterations in gene profiling reveals that activation of NF- κ B and its target genes is interdependent with a network of non-canonical NF- κ B and other key signal pathways altered in HNSCC, resulting in heterogeneity in the relative role and importance of these pathways in regulating gene signatures and the malignant phenotypes observed. It seems likely that miniaturization of phospho-proteomics of signaling and proteomics of target molecules using methods such as RPMA or chip based methods will proceed and be validated in larger clinical trials, and with different agents. These methods may facilitate selection of combinations of selective agents, and early evaluation of their molecular and cytotoxic activity in biopsies during the first weeks of treatment. The results of analysis of molecular targeting of EGFR and the proteasome indicate that these agents block only a portion of these signal networks in most HNSCC, suggesting that effective therapy will require a combination of agents targeted at these key pathways until such time as a smaller number of initiating events can be identified and targeted with precision.

As a next step, clinical trials combining agents against such targets have been designed. Trials using EGFR inhibitors or HDIs with proteasome inhibitor have been initiated. Combinations with HSP90 inhibitors and other agents targeting other tyrosine kinase receptors or downstream kinases are of interest. The future for more specific inhibitors of NF- κ B activation such as IKK inhibitors is uncertain. It is not known if their increased specificity and efficacy may result in greater clinical tumor activity or toxicity.

Executive summary

The role of the NF- κ B transcriptome and proteome as biomarkers in human head and neck squamous cell carcinomas

- NF- κ B is activated in the majority of HNSCC, and intensity of nuclear phosphorylated RELA p65 immunostaining has been shown to be a proteomic biomarker for risk of malignant progression of dysplasia and decreased survival in patients with HNSCC.

Constitutive activation of NF- κ B in head and neck cancer

- Nuclear activation of both canonical (NF- κ B1, RELA) and non-canonical (NF- κ B2 and RELB) subunits is observed in HNSCC
- Elevated proinflammatory and proangiogenic cytokine genes and proteins are detectable in cell lines, tumors and serum from patients with HNSCC

- Unique gene signatures and transcriptomes under regulation of NF- κ B are detected and have been examined in pathogenesis and as biomarkers of decreased survival in patients with HNSCC

NF- κ B regulated proteome and transcriptome as biomarkers and therapeutic targets in preclinical and clinical studies of head and neck cancer

- The NF- κ B regulated transcriptome and proteome in human and murine syngeneic SCC models represent up to ~60% of genes differentially expressed, and their genetic modulation inhibits tumorigenesis
- Promoter binding sites for other signal activated transcription factors are also highly represented, indicating that NF- κ B is part of a dysregulated signaling network

NF- κ B biomarkers in testing of newly developed therapeutic agents in preclinical *in vitro* and *in vivo* animal models

- Proteasome inhibitor bortezomib (VELCADE™/PS-341) inhibited NF- κ B, proliferation, cell survival, angiogenesis and tumorigenesis in preclinical studies
- Histone deacetylase inhibitors such as PXD 101 enhance NF- κ B acetylation and activation promoting resistance to this class of agents, and combination with proteasome inhibitor enhances cytotoxicity
- EGFR inhibitors such as Gefitinib/Iressa inhibit NF- κ B and co-activated MAPK, AKT, and STAT pathways in a subset of HNSCC

Clinical studies of newly developed therapeutic agents in head and neck cancer patients

- Phase I clinical trials with proteasome and EGFR inhibitors have demonstrated potential of using NF- κ B phospho-signaling and target proteins with Ki-67 and TUNEL assay in pre and on-treatment tumor biopsies for demonstrating molecular and cytotoxic response or unresponsiveness to targeted therapies
- Immunohistochemistry and reverse phase protein microarray methods hold promise for these applications
- Serum cytokines regulated by NF- κ B represent biomarkers of response, recurrence and survival in HNSCC

Conclusions

- Characterization of the NF- κ B transcriptome and proteome has led to the demonstration of frequent NF- κ B nuclear localization and target molecule expression as biomarkers associated with pathogenicity and survival of HNSCC
- Inhibition or decrease in these markers is observed with agents that inhibit NF- κ B or induce tumor responses, making them potentially useful in selection and monitoring of response to therapy.
- Activation of NF- κ B and its target genes is interdependent with a network of non-canonical NF- κ B and other key signal pathways altered in HNSCC, resulting in heterogeneity in the relative role and importance of these pathways in regulating gene signatures and the malignant phenotypes observed.
- Analysis of molecular targeting of EGFR and the proteasome indicate that these agents block only a portion of these signal networks in most HNSCC, suggesting that effective therapy will require a combination of agents targeted at these key pathways.

- Clinical trials combining agents against such targets mentioned above using EGFR inhibitors or HDIs with a proteasome inhibitor have been initiated.
- Combinations with HSP90 inhibitors and other agents targeting other tyrosine kinase receptors or downstream kinases are of interest.

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Figure 1.

NF-κB activation in cancer. NF-κB activation in cancer development is linked with chronic exposure to bacteria, certain viral products, chemical promoters, and carcinogens and reactive oxygen species (ROS), which cause repeated DNA damage. Induction also occurs in response to cytotoxic chemotherapy and ionizing radiation. A. Canonical NF-κB Pathway activation occurs in response these bacterial, viral, chemical or physical stimuli; by aberrant cytokine, integrin and growth factor ligand/receptor activation (e.g., TNFR, IL-1R, Integrin α6β4, EGFR, Her2/Neu and other serum factors); expression of activating fusion proteins following translocations (BCR-ABL, MALT1); or aberrant activation by intermediate kinases (e.g., PI3K, CK2, AKT). Intermediate kinases convey signals to the Inhibitor-κB complex formed by IKKα, β and γ, and IKKβ phosphorylates Inhibitor-κB, marking it for ubiquitiantion by E3 ligase βTrCP (SCF) and proteasome degradation. P105/RELA or cREL is processed to NF-κB1 (p50)/RELA or cREL heterodimers, which translocate to the nucleus, and bind promoters of genes regulating proliferation, apoptosis, migration inflammation, angiogenesis and innate immunity. B. Alternative or Non-canonical Pathway. The alternative pathway may be activated by other TNF family members via NIK, and involves IKKα/ IKKα homodimers, which activate NF-κB2/p100 for processing into p52/RELB heterodimers. The REL-B/p52 heterodimer then translocates into the nucleus to bind the promoter of genes whose products are important for the malignant phenotype in some cancers and B cell development and adaptive immunity. Abbreviations: NF-κB, nuclear factor κB; IKK, Inhibitor-κB kinase; NIK, NF-κB inducing

kinase; IL-1R, Interleukin 1 Receptor; EGFR, Epidermal Growth Factor Receptor; TNFR, Tumor Necrosis Factor Receptor; PI3-K, phosphatidylinositol 3-kinase.

Figure 2.

Co-activation of IL-1R/TNFR-NF-κB and EGFR/MAPK/STAT3/AP-1 pathways and proliferative, survival and angiogenesis factor genes in HNSCC may be inhibited by proteasome and EGFR inhibitors. Through transcription factor binding sites in the regulatory promoter region of genes, the MAPK-AP1, STAT3 and NF-κB pathways have been shown to independently promote activation of proliferative and prosurvival genes cyclin D1, IAP1 and BCL-XL, as well as angiogenesis factors IL-6, IL-8, VEGF and HGF, which contribute to the malignant phenotype and therapeutic resistance of HNSCC. Thus, targeting EGFR mediated activation of MAPK and STAT3 by EGFR tyrosine kinase or antibody inhibitors, and proteasome dependent activation of NF-κB by bortezomib, has potential for combinatorial activity against co-activated pathways important in HNSCC.

Figure 3. Hierarchical clustering analysis of differentially expressed genes in UM-SCC cells (reproduced from Yan et al, Genome Biol, 2007). 1011 differentially expressed genes were extracted from 24K cDNA microarray database, based on a two-fold and above difference among human normal keratinocytes (HKC), UM-SCC cells with different p53 status (t-test score at $P < 0.05$, two-tailed). The hierarchical clustering tree was generated using Java Treeview. Four HKCs were grouped on the left, and 5 UM-SCC cell lines with wild-type p53-deficient (wt p53-def) expression pattern were grouped together in the middle, and 5 UM-SCC cell lines with mutant (mt) p53 grouped to the right, respectively. Over-expressed genes are indicated by red and under-expressed genes by green; and the expression level is proportional to the brightness of the color (see color bar). **A**, entire hierarchical clustering tree included 3 up-regulated clusters A, B and C (including sub-clusters C1-3), and 3 down-regulated D, E and F; **B**, cluster A consisted of 34 genes; and **C**, cluster B consisted of 37 genes.

Figure 4.

Frequency of putative transcription factor binding sites (TFBS) in proximal regions of promoters (reproduced from Yan et al, Genome Biol, 2007). The promoter sequences were extracted from the over-expressed clusters A, B and C1-3 genes in UM-SCC cells using Genomatix Suite 3.4.1 (www.genomatix.de). The average length of these promoters was adjusted to approximately 600, including ~500 bp upstream and ~100 bp downstream from TSS. The promoter sequences from vertebrates represented 159,505 promoters, including 55,207 from human, 69,108 from mouse and 35,190 from rat in Genomatix promoter database (GPD). The *P* value of TFBS frequency in a given cluster was calculated by MatInspector of Genomatix Suite 3.4.1. * indicates the significantly increased frequencies of putative binding motifs on promoter regions of clustered genes when compared with the vertebrate promoters with a randomly drawn samples of the same size ($P < 0.05$). † indicates a significantly lower frequency of the AP-1 binding motif when compared with the vertebrate promoters.

Table 1

Signal pathways associated with NF-κB regulated genes in HNSCC

Tumor type ^a	Pathway	Genes ^b
All subgroups	Ephrin Receptor Signaling	<i>ANGPT1</i> ↓, <i>AXIN1</i> ↑, <i>CXCL14</i> ↑, <i>EFNB1</i> ↓, <i>EPHB2</i> ↑, <i>GNAI2</i> ↓, <i>GNAI5</i> ↓, <i>GNG12</i> ↓, <i>IL-8</i> ↑, <i>ITGA2</i> ↓, <i>PGF</i> ↓, <i>RAC2</i> ↓, <i>RHOA</i> ↓, <i>VEGFC</i> ↓
	ERK/MAPK Signaling	<i>DUSP4</i> ↓, <i>DUSP6</i> ↓, <i>ELF3</i> ↑, <i>ETS1</i> ↓, <i>ITGA2</i> ↓, <i>PIK3R3</i> ↑, <i>PLCG2</i> ↑, <i>PPP2R5B</i> ↓, <i>PPARG</i> ↑, <i>RAC2</i> ↓
	IL-6 Signaling	<i>IKBKE</i> ↑, <i>IL-1A</i> ↓, <i>IL-1B</i> ↓, <i>IL-1R2</i> ↓, <i>IL-1RN</i> ↓, <i>IL-6</i> ↑, <i>IL-6ST</i> ↓, <i>IL-8</i> ↑, <i>LBP</i> ↑, <i>MAP4K4</i> ↓, <i>TNFRSF1A</i> ↓
	Inositol Phosphate Metabolism	<i>ISYNA1</i> ↑, <i>ITPKA</i> ↑, <i>NEK2</i> ↑, <i>PIK3R3</i> ↑, <i>PIM1</i> ↑, <i>PLK1</i> ↑, <i>PRKCQ</i> ↓, <i>PLCD1</i> ↓, <i>PLCG2</i> ↑, <i>PRKX</i> ↓
	Leukocyte Extravasation Signaling	<i>ACTN3</i> ↓, <i>CD99</i> ↓, <i>CLDN7</i> ↑, <i>CXCL14</i> ↑, <i>CYBA</i> ↑, <i>GNAI2</i> ↓, <i>ICAM1</i> ↑, <i>IL-8</i> ↑, <i>MMP13</i> ↑, <i>PIK3R3</i> ↑, <i>PLCG2</i> ↑, <i>PRKCQ</i> ↓, <i>RAC2</i> ↓, <i>RHOA</i> ↓, <i>TIMP2</i> ↑, <i>VASP</i> ↓
	p38 MAPK Signaling	<i>DUSP10</i> ↑, <i>IL-1A</i> ↓, <i>IL-1B</i> ↓, <i>IL-1R2</i> ↓, <i>IL-1RN</i> ↓, <i>MAPKAPK3</i> ↓, <i>PLA2G4B</i> ↑, <i>TGFBR2</i> ↓, <i>TNFRSF1A</i> ↓
	TGF-β Signaling	<i>BMP4</i> ↓, <i>INHBA</i> ↓, <i>SERPINE1</i> ↓, <i>TGFBR2</i> ↓, <i>SMURF2</i> ↓, <i>VDR</i> ↓
	Wnt/β-catenin Signaling	<i>AXIN1</i> ↑, <i>CCND1</i> ↑, <i>DKK3</i> ↓, <i>PPP2R5B</i> ↓, <i>SFRP1</i> ↓, <i>SOX8</i> ↓, <i>SOX9</i> ↓, <i>TCF4</i> ↓, <i>TGFBR2</i> ↓
	Xenobiotic Metabolism Signaling	<i>ALDH1A2</i> ↑, <i>ALDH5A1</i> ↑, <i>ALDH1A3</i> ↑, <i>ALDH3B2</i> ↑, <i>CYP3A4</i> ↓, <i>FMO3</i> ↓, <i>IL-1A</i> ↓, <i>IL-6</i> ↑, <i>NOS2A</i> ↓, <i>NQO1</i> ↑, <i>PIK3R3</i> ↑, <i>PPARBP</i> ↓, <i>PPP2R5B</i> ↓, <i>PRKCQ</i> ↓, <i>SULT1A3</i> ↑
wt p53-deficient	Cell Cycle:G2/M Checkpoint	<i>CDKN1A</i> ↓, <i>PLK1</i> ↑, <i>RPS6KA1</i> ↓, <i>SFN</i> ↓, <i>TOP2A</i> ↑
	Neuregulin Signaling	<i>ADAM17</i> ↓, <i>ITGA2</i> ↓, <i>NRG2</i> ↓, <i>PDK1</i> ↑, <i>PICK1</i> ↓, <i>PRKCQ</i> ↓
	PPAR Signaling	<i>IL-1A</i> ↓, <i>IL-1R2</i> ↓, <i>IL-1RN</i> ↓, <i>IKBKE</i> ↑, <i>PPARBP</i> ↓, <i>PPARG</i> ↑
	Protein Ubiquitination Pathway	<i>BIRC2</i> ↑, <i>CDC20</i> ↑, <i>DOC1</i> ↓, <i>FBXW7</i> ↓, <i>NEDD4L</i> ↓, <i>PSMB10</i> ↑, <i>SMURF2</i> ↓, <i>UBE2H</i> ↓, <i>UBE2L6</i> ↑, <i>USP6</i> ↓
mt p53	GM-CSF Signaling	<i>AKT1</i> ↓, <i>CCND1</i> ↑, <i>CFS2</i> ↓, <i>ETS1</i> ↓, <i>PIK3R3</i> ↑, <i>PPP3CC</i> ↓
	IGF-1 Signaling	<i>AKT1</i> ↓, <i>CYR61</i> ↓, <i>IGFBP2</i> ↑, <i>IGFBP3</i> ↑, <i>IGFBP6</i> ↑, <i>IRS1</i> ↑, <i>PIK3R3</i> ↑, <i>RASA1</i> ↑, <i>SFN</i> ↓
	Integrin Signaling	<i>ACTG2</i> ↓, <i>ACTN3</i> ↓, <i>AKT1</i> ↓, <i>BCAR3</i> ↓, <i>DDEF1</i> ↓, <i>ITGA2</i> ↓, <i>ITGA5</i> ↓, <i>ITGB4</i> ↓, <i>LAMA3</i> ↓, <i>LAMB3</i> ↓, <i>LAMC2</i> ↓, <i>PIK3R3</i> ↑, <i>PLCG2</i> ↑, <i>RAC2</i> ↓, <i>RHOA</i> ↓, <i>RHOC</i> ↓, <i>TSPAN4</i> ↓, <i>TSPAN7</i> ↑, <i>VASP</i> ↓
	NF-κB Signaling	<i>AKT1</i> ↓, <i>BCL10</i> ↓, <i>IL-1A</i> ↓, <i>IL-1R2</i> ↓, <i>IL-1B</i> ↓, <i>MALT1</i> ↓, <i>MAP4K4</i> ↓, <i>PIK3R3</i> ↑, <i>PLCG2</i> ↑, <i>TNFRSF1A</i> ↓
	SAPK/JNK Signaling	<i>DUSP4</i> ↓, <i>DUSP10</i> ↑, <i>EDG5</i> ↓, <i>IRS1</i> ↑, <i>MAP4K4</i> ↓, <i>PIK3R3</i> ↑, <i>RAC2</i> ↓, <i>SH2D2A</i> ↓, <i>ZAK</i> ↓
	VEGF Signaling	<i>ACTG2</i> ↓, <i>ACTN3</i> ↓, <i>AKT1</i> ↓, <i>PGF</i> ↓, <i>PIK3R3</i> ↑, <i>PLCG2</i> ↑, <i>SFN</i> ↓, <i>VEGFC</i> ↓

Shown are signaling pathways associated with NF-κB target genes in UM-SCC cells using Ingenuity Pathway Analysis (IPA 5.0, www.ingenuity.com) with a significant enrichment ($P < 0.05$).

^aTumor type refers to subgroups with different p53 status which are associated with the major signal transduction pathways.

^bGenes refer to genes included in the pathway by IPA. ↓ and ↑ indicate up- and down-regulated gene expression with 2 or above fold changes.