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Review

Targeting receptor tyrosine kinase signaling: Avenues in the management of cutaneous squamous cell carcinoma

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SUMMARY

Non-melanoma skin cancer (NMSC) is the most frequently diagnosed cancer worldwide. Among the various types of NMSCs, cutaneous squamous cell carcinoma (cSCC) exhibits more aggressive phenotype and is also the second-most prevalent type. Receptor tyrosine kinases (RTK) triggers key signaling events that play critical roles in the development of various cancers including cSCC. Unsurprisingly, for this reason, this family of proteins has become the cynosure of anti-cancer drug discovery pipelines and is also being considered as attractive targets against cSCC. Though inhibition of RTKs in cSCC has yielded favourable results, there is still scope for bettering the therapeutic outcome. In this review, we discuss the relevance of RTK signaling in the progression of cutaneous squamous cell carcinoma, and observations from clinical trials that used RTK inhibitors against cSCC. Backed by results from preclinical studies, including those from our lab, we also give insights into the scope of using some natural products as effective suppressors of RTK signaling and skin carcinogenesis.

INTRODUCTION

Non-melanoma skin cancer (NMSC) is the most frequently diagnosed cancer worldwide with the highest incidence rate reported in Australia (>1000/100,000 persons) and the lowest in Africa (<1/100,000 persons).[1] Among NMSCs, basal cell carcinoma is the most prevalent form constituting 75% of the total NMSC cases followed by cutaneous squamous cell carcinoma (cSCC), which constitutes the huge majority of the rest of NMSC incidences.[2] The metastasis rate of NMSC is generally very low with cSCC showing the highest rate of about 3.7% of the total cases.[3]

Major factors that contribute to cSCC are ultraviolet (UV) rays, ionizing radiation, and chemical carcinogens. UV-induced development of intraepithelial lesions, called field cancerization, is the precursor ofa major proportion of cutaneous SCCs. These lesions consequently progress to actinic keratosis (AK), which shares similarity with field cancerization in exhibiting hyperchromatic and polymorphic nuclei with change in the nucleus to cytoplasmic ratio, and cell polarity.[4] However, AK shows unique properties like hyperkeratosis that often contain parakeratotic areas and lymphocytic infiltrate that might have implications in disease prognosis. Approximately 65% of cSCCs arise from these precancerous lesions.[5] The observed rate of transition of AK lesions into cutaneous squamous cell carcinoma is between 0.025% and 16%; however, it is not possible to identify the AK lesions that may progress into cSCC.[6,7] Studies focusing on the molecular mechanism of cSCC development have discovered the involvement of oncogenic pathways like Wnt/β-catenin, Notch, and receptor tyrosine kinase (RTK) signaling. RTK signaling has been shown to be a major contributor of cSCC development and is being targeted to treat cSCC.

RECEPTOR TYROSINE KINASE SIGNALING

Receptor tyrosine kinases comprise an extracellular domain, a transmembrane region, and an intracellular component which consist of a regulatory region, a tyrosine kinase domain and a carboxyl terminal tail. RTKs are activated by binding of ligands to specific receptors, leading to receptor dimerization/oligomerization. The alteration in their conformation facilitates phosphorylation and activation of tyrosine kinase domains.[8] Phosphorylation of RTKs triggers the activation of SH2 or PTB-domain containing proteins which binds to the phosphotyrosine residues of the receptors and propagates downstream signaling.

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Discovery of active kinases and their substrates in the cellular proteome is usually accomplished by kinomic analysis using peptide microarrays or by phosphoproteomic analysis by mass spectrometry. Aberrant cellular signaling can be a result of over-expression of genes that codes for these kinases or by mutations that produces constitutively active kinases. Genomic sequencing and transcriptomic analysis have discovered activating mutations and over-expression of multiple receptor tyrosine kinases in cSCC samples. Here, we elaborate the importance of receptor tyrosine kinases in cSCC and the scope of targeting these molecules for combating this cancer type.

Epidermal growth factor receptor (EGFR)

Activation of EGFR signaling is initiated by the binding of epidermal growth factor (EGF) to its receptor, EGFR, causing its dimerization followed by recruitment of effector proteins to the receptor and signal transduction from the membrane to the nucleus via cytoplasmic intermediate proteins. EGFR activation has a central role in enhancing the potential of cancer cells to evade apoptosis, proliferate and metastasize to form secondary tumors. Expression or activation of EGFR is implicated in the progression of cutaneous squamous cell carcinoma. A previous study has reported the hyper-phosphorylation of EGFR at Tyr-1068 and its down-stream effectors, ERK1/2 and Akt, in SCC compared to BCC and normal skin.[9] Consistent to this observation, over-expression of EGFR ligands, viz., amphiregulin, heparin-binding epidermal growth factor-like growth factor and transforming growth factor α, were observed in SCC samples.[10] However, the frequency of EGFR mutation in cutaneous SCC is low as indicated by a study conducted in a German cohort of SCC patients.[11] Apart from these clinical observations, experimental evidence reveals the crucial role of EGFR and associated signals in the progression of SCC. In animal models, over-expression of TGF-α induced hyperplasia, hyperkeratosis papilloma, and SCC.[12] Furthermore, EGFR expression was found to be an essential factor for the basal proliferation of skin tumor cells in murine models.[13] Another study reported that SMAD-7, an inhibitor of TGF-β, augmented tumor promotion through activation of EGFR signaling and enhancing DNA repair in murine models of skin carcinogenesis.[14] The proliferation of keratinocytes *in vitro* is dependent on EGFR signaling as abrogation of EGFR function results in cell-cycle arrest at different stages depending on the culture conditions.[15] Apart from ligand binding and mutation, activation of EGFR can also be triggered by exposure of skin to UV which accomplishes EGFR phosphorylation via ROS-dependent inactivation of tyrosine kinase phosphatases that maintains EGFR-tyrosine kinase phosphorylation at a basal level.[16] Transcriptome sequencing has identified a fusion gene, EGFR-PPARGC1A in cutaneous squamous cell carcinoma cells.[17] This fusion leads to the constitutive phosphorylation of tyrosine residues and elevates the phosphorylation of wild type-EGFR by causing receptor dimerization. Functionally, EGFR-PPARGC1A enhances the proliferative capacity of SCC cells as CRISPR/cas9-mediated knockdown of the gene in the SCC cell line, A431 lead to a decrease in cell number. EGFR signaling contributes to epithelial to mesenchymal transition of SCCs. EGF-induced activation of EGFR leads to ERK1/2 mediated activation of β-catenin and consequently, the transition from an epithelial to a mesenchymal phenotype.

Fibroblast growth factor receptor (FGFR)

Fibroblast growth factor receptor (FGFR) is over-expressed in cSCC samples when compared to normal skin and pre-malignant lesions[18]; moreover, cSCC cells also over-express FGF10, an FGFR binding ligand. Inhibition of FGFR by pan-FGFR inhibitor, AZD4547, decreases tumor growth in immunocompromised mice injected with SCC12 cells. The role of FGFR signaling in cSCC is underlined by studies that showed activation of FGFR as an alternate mechanism to activate down-stream signaling following inhibition of EGFR.[19] Contrary to this observation, another study discovered the role of FGFR3-FOXN1 loop in inducing differentiation of cSCC cells.[20] In addition, human skin grafts expressing active mutant FGFR3contributesto epidermal hyperproliferation but does not lead to SCC formation.[21] However, Ser249Cys substitution in the extracellular domain of FGFR3 in the basal cells of the epidermis results in formation of benign skin tumor[22]; moreover, gain of function mutations of FGFR3 were found in metastatic cutaneous squamous cell carcinoma.[23] The involvement of extrinsic factors and the modulation of the tumor cell transcriptome/proteome while transitioning through each stage of tumorigenesis might dictate the tumor burden and could be the reason for these contradicting observations.

Vascular endothelial growth factor receptor (VEGFR)

Signaling through vascular endothelial growth factor receptors (VEGFR) is another contributor to skin carcinogenesis. VEGFR signaling is initiated by the binding of vascular endothelial growth factor VEGF to VEGFR. SCC cell lines and human skin tumor cells display elevated expression of VEGF.[24] UV-exposure

to skin elevates the expression of VEGF, which is directly correlated with angiogenesis and tumor progression. VEGF over-expressing SCC-13 cells form invasive and highly vascularized tumors in immunocompromised mice.[25] Besides being a critical regulator of angiogenesis, VEGF contributes to autonomous cell proliferation.VEGFR-1 knockout K5-SOS mice that have constitutively active ras, have been reported to show low tumor burden, though the extent of angiogenesis remains unaffected. Activation of VEGFR-1 is essential for the maintenance of hair follicle stem cell population, which is a key driver of skin carcinogenesis.$CD34^+$ SCC cells in mouse have a higher expression of VEGF compared to $CD34^-$ tumor cells. Likewise, over-expression of VEGF in epidermal cells elevates $CD34^+$ stem cell population whereas DC101-mediated suppression of VEGFR2 signaling leads to reduction of the microvascular density and stem cell pool. Here, deletion of Nrp1, a VEGF co-receptor, also leads to a reduction in the stem cell population.[26] Apart from affecting the stemness and proliferation of epithelial cells, activation of VEGF receptors can affect the survival of keratinocytes. Exposure of keratinocytes to UV-light increases the expression of VEGF receptors viz, VEGFR1 and VEGFR2 via induction of oxidative stress. In keratinocytes, VEGF can suppress UV-induced apoptosis.[27]

AXL receptor tyrosine kinase (AXL)

Cutaneous squamous cell carcinomaexhibits over-expression of AXL as evidenced by microarray analysis of pre-malignant and SCC cells.[28] Depletion of AXL leads to increased sensitivity of SCC cells toward UV-induced apoptosis through activation of the intrinsic apoptotic pathway.[29] The induction of apoptosis is accompanied by down-regulation of Akt. However, here, knockdown of AXL did not have any significant impact on the proliferative potential of the cell. In another study, depletion of AXL increased cell-cell adhesion with concomitant decrease in Wnt and TGFβR signaling while up-regulation of Axl leads to increased stemness and drug resistance.[30]

Insulin growth factor receptor (IGFR)

IGFR signaling exhibits both tumor suppressive and tumor promoting potential depending on the cellular context. In keratinocytes, IGFR signaling triggers growth arrest on exposure to UV-radiation. In normal human skin, IGFR signaling in keratinocytes is triggered in a paracrine manner wherein IGF1 released by dermal fibroblasts initiates IGFR activation in keratinocytes. Skin devoid of IGF1 does not lead to growth arrest on exposure to UV, thus leading to tumorigenesis.[31] However, in the case of cSCC, IGFR signaling contributes to cell survival and proliferation. Multiple studies have shown the efficacy of targeting IGFR with EGFR to improve the treatment outcome in cSCC. A12, an inhibitor of IGFR, has been shown to suppress angiogenesis and cell proliferation.[32] Similarly, picropodophyllin, an IGFR inhibitor, was found to exert tumor suppressive effect against xenograft models of SCC.[33]

EPH receptor B4 (EPHB4)

EPHB4 is a receptor tyrosine kinase belonging to erythropoietin-producing hepatocellular family. The expression of EPHB4 and its ligand, ephrin B2 is inversely correlated with differentiation of cSCC.[34] Knockdown of EphB2 suppressed vascularization and progression of cSCC in xenograft models. Down-regulation of EphB2 was accompanied by decrease in expression of genes associated with invasion and migration.[35]

Hepatocyte growth factor receptor (MET)

cSCC shows over-expression of MET, a receptor tyrosine kinase that binds to hepatocyte growth factor (HGF).[36] Aberrant activation of MET contributes to increased tumor development in Tpl2 depleted skin. Tpl2 depleted mice displays more robust tumor yield compared to wild type mice on treatment with 7,12-Dimethylbenz[a]anthracene (DMBA) and Phorbol 12-myristate 13-acetate (PMA). However, when capmatinib, a MET inhibitor was administered to Tpl2 depleted mice, a reduction in tumor burden was observed and none of the tumors acquired a malignant phenotype.[37]

Erb-B2 receptor tyrosine kinase 2 (ERBB2)

Constitutive activation of ERBB2, a receptor tyrosine kinase structurally related to EGFR, in epidermis leads to epidermal hyperproliferation and tumor development.[38] Similarly, another study showed that ERBB2 is essential for skin tumorigenesis though they are dispensible for maintenance of the epidermis.[39] Exposure of skin to UV-radiation elicits ERBB2 activation, which promotes inflammation and cell proliferation, and is essential for cell survival on UV-exposure.[40]

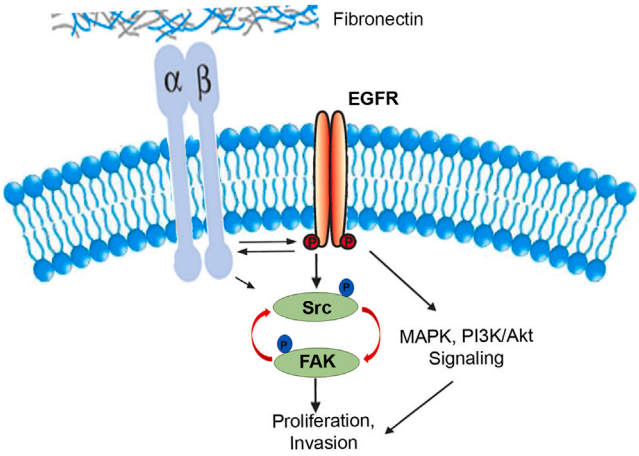

Figure 1. Diagrammatic representation of the activation of receptor tyrosine kinase signaling

Signaling activators like UV-rays, vascular endothelial growth factors and fibroblast growth factors activates receptor tyrosine kinases and downstream signaling molecules in the MAPK, PI3K/Akt and STAT pathways. This leads to activation of transcription factors which enhances the transcription of their target genes.

SIGNALING MECHANISMS OPERATING DOWN-STREAM TO RECEPTOR TYROSINE KINASES

Ras/mitogen-Activated protein kinase (MAPK) and ras/phosphoinositide-3-kinase (PI3K)/Akt

Similar to other cancers, cutaneous SCCs exhibit activating mutation and amplification of the Ras oncogene, which codes for a family of GTP-binding proteins that operates down-stream to receptor tyrosine kinases. Around 20% of the SCCs have mutated *HRAS* genes.[41] *HRAS* mutations are located at codons 12, 13 and 61. As seen in human SCCs, tumors induced by the application of DMBA and PMA on mouse skin also exhibit mutation at *HRAS* at codon 61. Activation of Ras consequently activates its down-stream proliferative signaling molecules, viz., Raf/Mek/Erk1/Erk2 and PI3K/Akt that leads to the development of SCC. We have shown that application of DMBA/PMA induces activation of MAPK signaling in mouse skin.[42] *HRAS* mutation triggers constitutive activation of cell survival and proliferative signals and consequent cell proliferation irrespective of the activation status of receptor tyrosine kinases.

Activation of Ras leads to the activation of the PI3K/Akt or MAPK signaling. cSCC samples shows higher activation of PI3K/Akt signaling which is not driven by hotspot mutations.[43] Multiple preclinical studies point toward a strong pro-tumorigenic role for Akt in skin cancer. Hair follicle stem cells expressing a constitutively active form of Akt shows enhanced ability to exit quiescence and are more sensitive toward proliferative stimuli.[44] In agreement to this observation, over-expression of Akt in epidermis increases the tumor burden induced by application of DMBA/PMA.[45] In addition, hyperactivation of mTOR, which operates downstream to Akt, is also observed in cSCC.[46] Exposure of normal skin to UV-light activates Akt/mTOR as well as MAPK signaling (Figure 1). Skin exposed to UV-light showed sustained increase in the phosphorylation of ERK1/2.[47]

Signal transducer and activator of transcription 3 (STAT-3)

STAT3 is one of the main downstream effectors of receptor tyrosine kinase signaling. These belong to a highly conserved family of proteins comprising seven members, STAT1 to STAT4, STAT5a, STAT5b, and STAT6. In resting cells, STATs are generally located in the cytoplasm in their inactive state. Phosphorylation of specific tyrosine residue is an essential step in STAT activation. Once activated, STAT dimerizes to other STATs by reciprocal SH2 phosphotyrosine interaction, leading to its translocation into the nucleus followed by its binding to the specific enhancer elements for initiation of transcription. Activation of STAT-3 has implications in cancer cell proliferation, invasion, and metastasis. Human SCCs display hyper-phosphorylation of STAT-3 when compared to normal skin and is inversely correlated with cellular differentiation levels.[48] Phosphorylation of STAT3 is correlated with that of *E*-cadherin. Multiple studies in mice models of skin carcinogenesis have highlighted the crucial role of STAT3 in skin cancer. SCCs from transgenic mice expressing a constitutively active STAT-3 (K5.Stat3C) show low expression of E-cadherin and high

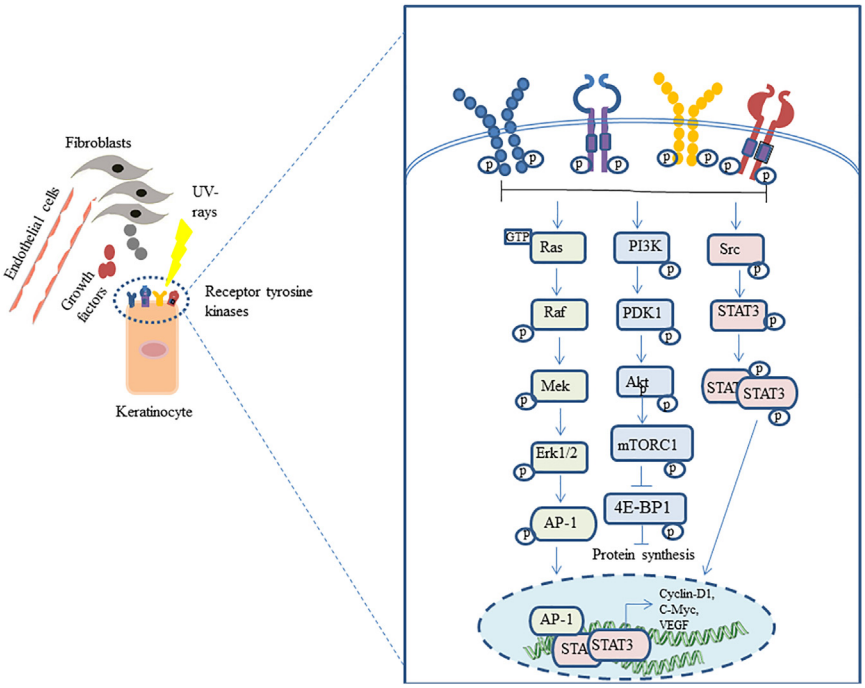

Figure 2. Diagrammatic representation of β-catenin activation via EGFR signaling

β-catenin exist in the cell membrane and cytoplasm. In the membrane, it is bound to E-cadherin and in cytoplasm, it is bound to a complex comprising Axin, GSK-3β, APC and CK-1. This destructive complex constantly phosphorylates β-catenin leading to its proteosomal degradation.Binding of EGF to EGFR causes the activation of ERK1/2 which phosphorylates CK-2 which subsequently phosphorylates α-catenin thus releasing its inhibitory effect on β-catenin and causing its translocation to the cytoplasm. Activation of EGFR also results in phosphorylation of Akt which phosphorylates and inactivates GSK-3β. This hurdles the phosphorylation of β-catenin, prevents its degradation, thus leading to its accumulation in cytoplasm and subsequent translocation to the nucleus where it promotes the transcription of pro-tumorigenic genes.

expression of Twist, thus perpetuating epithelial to mesenchymal transition.[49] STAT-3 deficient mice areless resistant to UV-induced apoptosis and shows reduced skin thickeningupon exposure to UV.[50] In agreement to this observation, K5.Stat3C mice were resistant to UV-induced apoptosis and are highly susceptible to UV-driven skin carcinogenesis.[51]

PATHWAY CROSSTALKS

β-catenin signaling

The canonical Wnt signaling culminates in the activation of β-catenin, a constituent of the cell adhesion complex also known to have transcriptional activity. In normal state, β-catenin is part of the cell adhesion complex and is bound to E-cadherin in the plasma membrane. Binding of Wnt to Frizzled leads to the disassembly of the trimeric complex comprising APC,β-catenin, and GSK-3, leading to the activation of β-catenin (Figure 2). As in many other cancers, Wnt signaling is dysregulated in a good proportion of cutaneous SCCs. The earliest study that reported this dys-regulation in SCC cells found frequent amplification of chromosome arms 7q,8q,11q, and 17q which has genes encoding Wnt or its receptor, Frizzled.[52] More evidence for the over-expression of Wnt signaling associated genes in cSCC came from gene expression analysis that reported an increase in the mRNA levels of WNT ligands and a down-regulation of Wnt inhibitors like secreted FZD-related proteins (SFRPs) that abrogates Wnt-Frizzled interaction.[53,54] Apart from the canonical Wnt signaling, activation of β-catenin is also achieved by receptor tyrosine kinase activation. We and other research groups have shown that treatment of A431 cells with EGF results in the activation of β-catenin.[42,55] Down-regulation of E-cadherin by the activation of EGFR leads to nuclear localization of β-catenin, which is often correlated with loss of cell differentiation, and elevation of invasive and metastatic capability of cancer cells.[56]The importance of β-catenin in SCC was proved using murine models of skin carcinogenesis. Exposure of mouse or human keratinocytes to UV elevated the expression and the

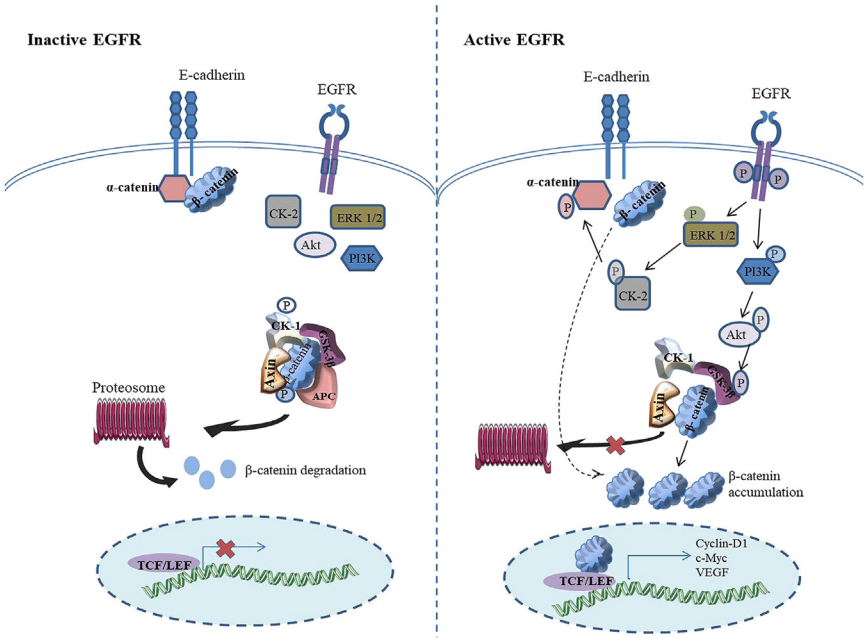

Figure3. Diagrammatic representation of Integrin-EGFR crosstalk

Activation of Integrin by interaction with fibronectin leads to activation of EGFR and downstream signaling, leading to cell proliferation and invasion. Conversely, activation of EGFR leads to activation of integrin and downstream Src and FAK activation.

transcriptional activity of β-catenin through a cox-2 dependent pathway.[57] REG-γ, a proteasome activator, augments the stability of β-catenin by curbing GSK-3β mediated phosphorylation of β-catenin while promoting DMBA/TPA-induced skin carcinogenesis.[58] The importance of β-catenin in the maintenance of cancer stem cells has also been investigated. Malanchi et al., showed the role of β-catenin in supporting the proliferation of hair-follicle stem cells in murine models of skin carcinogenesis.[59] Here, mice devoid of β-catenin were highly resistant toward DMBA/PMA-induced skin carcinogenesis.

Integrin signaling

Multiple studies have reported EGFR-integrin crosstalk and their physiological implications. Integrins are heterodimeric membrane-bound proteins that function as cell adhesion molecules by binding to extracellular matrix proteins. Integrin-ECM interaction triggers multiple intracellular signals and influences a variety of cellular properties like proliferation, migration, and differentiation. Integrin triggers proliferation of epithelial cells by activating EGFR.[60] Conversely, activation of EGFR can regulate integrin activation and spatial organization of adhesion molecules[61] (Figure 3). Similarly, IGF-1R interacts with and stabilizes β-integrins, and contributes to cell migration.[62] Microarray based gene expression analysis of normal skin and cutaneous SCC shows a highly significant differential expression of genes that function in integrin signaling.[63] The importance of integrin signaling in cSCC has been demonstrated in animal models. Integrin α3β1 plays a vital role in skin carcinogenesis as Itga3 KO mice are resistant to skin tumor development induced by the application of DMBA and PMA. Itga3 KO mice displayed increased epidermal turnover and hence lost DMBA initiated-label retaining stem cells that are critical for the development of skin carcinogenesis.[64] Integrin α6β1 was also found to play a key role in skin carcinogenesis. Integrin α6β1 is a crux determinant of the potency of SCC stem cells to give rise to secondary tumors following implantation into immunocompromised mice.[65]

TARGETED THERAPIES

Considering the prime role of the described pathways in the progression of cutaneous squamous cell carcinoma, multiple approaches targeting these have emerged. In clinical trials, some of these approaches have become successful to some extent in restricting or managing cSCC though these interventions elicited significant sideeffects.

Anti-EGFR monoclonal antibodies

As discussed earlier, EGFR signaling plays a major role in the progression of cutaneous squamous cell carcinoma, and hence, targeting EGFR is a logical approach in the management of cSCC. Cetuximab, an FDA-approved human–murine anti-EGFR monoclonal antibody binds to the second (L2) domain of EGFR thus facilitating receptor internalization, thereby blocking receptor-ligand interaction and consequent downstream signaling. The first clinical study to assess the effect of EGFR inhibition on cSCC was done on two elderly patients with recurrent, in-transit metastatic cSCC. The patients were treated with cetuximab and reported good response to the treatment. One of the patients reported complete response by 16 weeks of treatment while the other showed near-complete response by 12 weeks.However, grade 3 acneiform rash was reported in one patient which necessitated 2 weeks break from treatment.[66] In another clinical study, thirty-six patients with unresectable cSCC were administered with cetuximab. The primary endpoint for the study was disease control rate (DCR) at 6 weeks. Secondary endpoints were best response rate, overall survival, progression-free survival and toxicity assessment. The study showed that Cetuximab could accomplish a disease control rate of 69%.[67] The best responses were two complete response and eight partial response, and no drug-associated mortality was observed. However, treatment with cetuximab induced significant side effects such as acne-like rashes, infusion reactions, and interstitial pneumopathy. Another study using cetuximab was done on an elderly patient who did not respond to platinum-based therapies. The study reported that cetuximab coupled with a daily dose of orally administered curcumin phospholipid could exert significant anti-cancer effect without recurrence for 11 months and induced only mild skin-related toxicity.[68] A study using panatimumab, another monoclonal antibody against EGFR, was conducted on patients having advanced cutaneous squamous cell carcinoma. The primary endpoint was the best overall response rate. Secondary endpoints were safety, toxicity and progression-free survival. The overall response rate for therapeutic intervention was 31%.[69] Here, skin toxicity was observed in 25% of the subjects.

Tyrosine kinase inhibitors

Tyrosine kinase inhibitors have also been proved to be effective against cSCC. Gefitinib, an anilinoquinazoline derived low-molecular-weight EGFR tyrosine kinase inhibitor with selective tyrosine kinase activity, is effective against cutaneous SCC. A phase-II clinical trial was done to assess the effectiveness of neoadjuvant gefitinib administered before surgery and/or radiotherapy. In this study, gefitinib was active and well-tolerated, did not interfere with the treatment modalities, and yielded a complete response rate of 18% and partial response of 27.3%.Toxicity evaluation showed that 59.1% of the subjects experienced grade 2 or grade 3 toxicity and 9.1% of the subjects had to discontinue gefitinib administration because of adverse events.[70] A single-arm phase-II clinical trial conducted on patients with recurrent or metastatic cSCC showed that erlotinib, another tyrosine kinase inhibitor, yielded only an overall response rate of 10% with all of them being partial responses. In this study, adverse events were observed in all patients; however, most of the subjects showed only grade 1 and 2 toxicities with the majority being acneiform rash (64%) followed by fatigue in 46% of subjects.[71]

Potential RTK inhibitors for clinical trials

FGFR inhibitors, erdafitinib, and pemigatinib are being used to treat urothelial carcinoma and unresectable cholangiocarcinoma respectively.[72,73] The observation that metastatic cSCC harbors gain of function mutation in *FGFR* points to the possibility of repurposing these inhibitors for treatment of advanced cSCC.

Bemcentinib an AXL inhibitor in combination with an anti-PD-(L)1 agent has been found to be efficient in the treatment of STK11 altered advanced/metastatic non-small cell lung carcinoma (NSCLC) patients.[74] Over-expression of AXL in SCC increases the resistance of SCC cells to apoptosis and hence AXL inhibitors may prove to be effective in the treatment of cutaneous SCC.

Cabozantinib, a tyrosine kinase inhibitor which blocks MET, VEGFR2 and RET has shown substantial anticancer activity and has gained FDA approval for the treatment of medullary thyroid cancer and advanced renal cell carcinoma.[75,76] Similarly, crizotinib, a multi-target oral tyrosine kinase inhibitor of c-MET, ROS proto-oncogene 1 (ROS1), and anaplastic lymphoma kinase (ALK) has shown considerable activity in metastatic ALK-fusion NSCLC.[77] Emerging preclinical studies give evidences that a combinatorial approach using MET inhibitors and immunotherapy would serve as an effective therapeutic intervention in cSCC.

Table 1. Summary of RTK inhibitors and their known targets

Target	Drug	Clinical Indications
FGFR	Erdafitinib	Urothelial carcinoma[72]
	Pemigatinib	Unresectable cholangiocarcinoma[73]
AXL	Bemcentinib	NSCLC[74]
MET, VEGFR2 and RET	Cabozantinib	Medullary thyroid cancer & Advanced renal cell carcinoma[75,76]
c-MET, ROS, and ALK	Crizotinib	ALK-fusion NSCLC[77]
IGF1	Dasatinib	Chronic myeloid leukemia[78]
EPH	Ganitumab	Ewing sarcoma[79]
VEGFR	Axitinib	Renal cell carcinoma[80]
	Bevacizumab	Epithelial ovarian, fallopian tube, or primary peritoneal cancer[81]

Drugs targeting IGF1 and EPH receptors, such as dasatinib and ganitumab are being used in the treatment of chronic myeloid leukemia and Ewing sarcoma respectively.[78,79] Potent inhibitors of VEGFRs 1–3 such as axitinib and bevacizumab are being used in the treatment of renal cell carcinoma and epithelial ovarian, fallopian tube, or primary peritoneal cancer respectively.[80,81] As aberrant activation and regulation of these receptors have been extensively observed in development of cSCC. The major RTK inhibitors have been summarized in Table 1 and the use of these inhibitors might be an efficient strategy for the treatment of cSCC.

EXPERIMENTAL MODELS FOR STUDYING cSCC

Currently, there exist an array of *in vitro*, *in vivo* and *ex vivo* models which may be used to evaluate the efficacy of novel therapeutic modalities against cSCC. *In vitro* models comprise of studies using cell lines and primary cells that are cultured from patient derived tumor tissues. Genetically engineered mouse models, chemically induced carcinogenesis models and non-murine *in vivo* models such as zebrafish, are being used for cSCC studies. Recently, various *ex vivo* approaches such as 3D spheroid and organoid culture, skin reconstructs, and tissue explant culture have been developed to complement in vivostudies in murine models. Some of the most widely studied experimental models of cSCC are discussed below:

In vitro models

In vitro studies include the use of cSCC cell lines that are of human (for example, A431, SSC 12/13, HSC 1/5) and murine (for example, XL50) origin for evaluating the therapeutic efficacy of treatment modalities, and for studying the molecular mechanisms underlying drug resistance and cancer progression. Primary cSCC cells that have been isolated from patient samples can also serve as suitable *in vitro* cSCC models. However, *in vivo* models of cSCC provide a better understanding of the tumor microenvironment, immune response and metabolism.

Ex vivo 3D models

Various 3D culture models such as spheroids, organoids, and skin reconstructs have been developed and are currently used to study the molecular aspects related to cSCC[82,83]. Spheroids are the basic 3D culture model in which tumoral cells are cultured either without letting them adhere to the plastic surface or are cultured within synthetic matrices or scaffolds. Organoids comprise multiple cells types that organize into an organ-like structure *in vitro*. Skin reconstructs represent a co-culture model in which tumor cells and stromal cells are cultured together. These models mimic the human disease pathology and are hence suitable for drug development and chemoresistance studies.

In vivo murine models

Chemical-induced carcinogenesis model

DMBA (9,10-dimethyl 1,2-benzanthracene), a highly potent mutagen and TPA (12-*O*-tetradecanoyl phorbol-13-acetate), a non-mutagenic tumor promoter, are commonly used to establish multistage chemical-induced skin cancer in mice. As a result of this treatment, mice develop tumors having mutant *HRAS*.[84]

Table 2. Molecular targets of the phytochemicals in receptor tyrosine kinase signaling

Compounds	Targets
Quercetin	PI3K, Raf, MEK[86,87]
Kaempferol	Src, RSK, MSK1[90,91]
Curcumin	EGFR[93]
Apigenin	Src[100]
Resveratrol	p110α, p110β[107]
Luteolin	PKC[112]
EGCG	EGFR, IGFR[122,123]
Tryptanthrin	VEGFR2[121]

This skin carcinogenesis model is predominantly used to study the mechanistic aspects of epithelial carcinogenesis, and for testing the efficacy of anti-cancer drugs at impeding or blocking tumor initiation, promotion, and progression.

Cell line derived xenograft models

Here, tumors are developed by injecting human cSCC cell lines such as SCC13 and A-431 in immunocompromised mice.[85]

Genetically engineered mouse models (GEMMs)

GEMMs are developed to harbor mutations in tumor suppressor genes or oncogenes such as *TP53*, *BRAF*, *KRAS*, and *HRAS* that function as key drivers of cSCC.[86] The GEMMs are developed either individually or in combination with chemical or environmental carcinogenesis models or with PDX models.

Patient derived xenograft models

PDX models involve grafting of patient tumor tissue explants in immunocompromised mice. These advanced xenograft models help in predicting patient response to targeted therapies far better than murine xenograft models. Though PDX models are being extensively used to study other tumor models, currently there is only one study that used PDX model to study cSCC.[87]

In vivo non-murine models

Recently, transgenic zebrafish strains containing mutated *TP53* or *BRAF* or *HRAS* are being used to study cSCC to study the efficacy of drugs in ameliorating tumor progression.

NATURAL PRODUCTS AS INHIBITORS OF RECEPTOR TYROSINE KINASE SIGNALLING

Several bioactive compounds from natural sources have been shown to be very effective in suppressing carcinogenesis. A huge proportion of currently used chemotherapeutic agents are derived from natural sources. Preclinical studies on the efficacy of bioactive natural products have been undertaken to make a preliminary assessment of their potency against cSCC, and some of them have been used in clinical trials to test their efficacy against cSCC. Many of these compounds are known to down-regulate receptor tyrosine kinase signaling either by direct inhibition of the signaling components or by inhibiting pathways that crosstalk with this signaling pathway thereby suppressing cSCC.The studies on major phytochemicals inhibiting the receptor tyrosine kinase have been summarized below and their molecular targets are listed in Table 2.

Quercetin

Quercetin is a flavanoid found in apples and berries. It exhibits inhibitory activity against EGFR. Quercetin suppresses the phosphorylation of EGFR in the human epidermoid squamous cell carcinoma cell line, A431[88]; moreover, quercetin is an inhibitor of PI3K, Raf, and Ras which operate downstream to EGFR.[89,90] Although it is effective in inhibiting signals that drive skin carcinogenesis, its efficacy against non-melanoma skin cancer has not been explored well.

Kaempferol

Kaempferol is a tetrahydroxyflavone found in beans, kale etc., and is known to exhibit potent chemopreventive activity against cancers of various origin. One of the major targets of kaempferol is src, a protein kinase acting down-stream to G-protein coupled receptors (GPCRs). Multiple studies have demonstrated the activation of MAPKs via src.[91,92] Kaempferol suppresses UV-induced expression of cyclooxygenase-2 (Cox-2) in skin via attenuation of Src activation by competitively binding to the ATP-binding domain of Src.[93] Another study has demonstrated that kaempferol directly interacts with the ATP-binding sites of RSK and MSK1, which operates downstream to MAPK signaling, thereby suppressing UV-induced skin cancer.[94]

Curcumin

Curcumin inhibits the activation of EGFR via two mechanisms: inhibiting the kinase activity of EGFR intracellular domain, and modulating the EGFR binding environment in the cell membrane, thereby reducing receptor dimerization on ligand binding.[95] Curcumin has been shown to attenuate DMBA/PMA-induced skin cancer in normalSwiss albino mice[96] and in transgenic mice over-expressing IGF-1 in epidermis.[97] Here, curcumin has suppressed the activation of IGF-1R, Akt, S6K, and 4EBP1. In another study, treatment of mouse skin with curcumin before application of PMA suppressed epidermal hyperproliferation by attenuation of MAPKs and associated transcription factors via suppression of Protein Kinase C (PKC) activation.[98] Studies have also demonstrated that curcumin can abrogate EGFR-integrinα6β4 interaction in skin cancer cells.[99] Curcumin exhibits poor pharmacokinetic properties. We have earlier reported the efficacy of nanoformulations of curcumin in improving its pharmacokinetic properties.[100,101] Considering the effectiveness of curcumin against cSCC, it might be interesting to see the effectiveness of nanoformulation of curcumin against cSCC. A clinical trial to assess the effectiveness of curcumin against skin cancer showed that curcumin can accomplish very significant symptomatic relief, and reduction in pain and tumor size was observed in 10% of the subjects.[102]

Apigenin

Apigenin, a flavonoid found in fruits and vegetables, has potent anticarcinogenic activity. The compound directly binds to src kinase and exerts tumor suppressive effect against UV-induced skin cancer by hindering the activation of this protein.[103] The compound also suppressed PMA-induced expression of COX-2 in keratinocytes by blocking the activation of Akt and T-cell-restricted intracellular antigen 1-related protein (TIAR).[104]

Resveratrol

Resveratrol exhibits chemotherapeutic, chemopreventive, and chemosensitizing potential against cancers of multiple origins. Oral administration of resveratrol suppresses UV-induced skin tumorigenesis in mice.[105] In these models, resveratrol suppressed the phosphorylation of Akt and CREB, two regulators of TGF-β2 expression. Another study has demonstrated that resveratrol can inhibit p110α and p110β, two catalytic subunits of PI3K, by non-covalently binding to the ATP-binding sites of the proteins.[106] These evidences explain how resveratrol inhibits Akt signaling in multiple cell lines, and exert chemopreventive activity. Though previous studies indicate the possibility of using resveratrol against skin cancer, there are concerns regarding its photostability. Exposure of the compound to UV for longer duration may alter its structure. Researchers have improved the photostability of the compound by incorporating it into lipid-core nanocapsules.[107] In a clinical study, topical application of an antioxidant concoction containing resveratrol, baicalin and vitamin E on photodamaged skin showed significant improvement of skin elasticity, firmness, laxity and hyperpigmentation. However, clinical trials on the effectiveness of resveratrol against cSCC are lacking.[108]

Luteolin

Luteolin is a flavanoid found in cereals, onion leaves, broccoli, carrots, cabbages, and so on. Previous studies have shown its activity as a broad-spectrum anti-cancer agent. Luteolin shows inhibitory effect against PKC in both cellular and cell free systems.[109] Treatment of A431 cells with luteolin hurdles the activation of EGFR, suppresses the expression of Matrix Metalloproteinases-2 and 9, and induces apoptosis.[110]Consistent with this observation, another study has reported that luteolin can abrogate the activation of Src and STAT3 in A431-III cells, and could suppress invasive and migratory potential of these cells.[111]

Epigallocatechin gallate (EGCG)

EGCG, a phytochemical found in green tea, has shown very appreciable therapeutic efficacy against preclinical models of skin cancer. Application of EGCG suppresses UV-induced skin cancer in mice models.[112] Green tea extract enriched with catechin and EGCG has been shown to suppress 12-*O*-Tetradecanoylphorbol-13-acetate (TPA)-induced activation of MAPK in mouse skin.[113] Because the hydrophilic nature of EGCG challenges its efficient permeation into skin, researchers have attempted to develop vehicles to counteract this limitation. A self-emulsifying drug delivery system containing lipids and macadamia oil accomplished more efficient transdermal delivery of EGCG compared to aqueous formulation.[114] A clinical trial was conducted to assess the potency of EGCG in subsiding actinic keratoses. In this study, 51 subjects applied EGCG on one forearm having actinic keratoses and placebo in the other for 12 weeks. Though a decrease in severity of the condition was observed during the study in both groups, no significant difference was observed between the two groups[115]

Tryptanthrin

Tryptanthrin is an indoloquinazoline alkaloid first isolated from *Isatis tinctoria*, a medicinal plant mainly located in temperate climate zones. We isolated an anti-cancer fraction, DW-F5, which contains tryptanthrin as the majorbioactive molecule, from the leaves of *Wrightia tinctoria*.The fraction exhibited excellent efficacy against skin cancer, and tryptanthrin, isolated from this fraction, was found to be have impressive cytotoxic effect against melanoma.[116] Another study conducted in our lab revealed that, tryptanthrin is very potent in suppressing DMBA/PMA-induced skin carcinogenesis. Here, tryptanthrin suppressed the promotion stage of skin carcinogenesis by down-regulating MAPK and β-catenin activation.[42] One of the molecular targets of tryptanthrin is VEGFR2(117). In endothelial cells, tryptanthrin suppresses VEGFR-2/MAPK signaling, thus inhibiting angiogenesis.[117]

Conclusion

Cutaneous squamous cell carcinoma is the second most frequent form of non-melanoma skin cancer, and is one of the most aggressive type of NMSCs. Though the mortality rate of cSCCs is very low compared to cancers of other epithelial tissues, they pose a significant threat to the quality of life and impose a huge financial burden on the patients. Signal transduction via receptor tyrosine kinases triggers the activation of a multitude of proteins that contributes to skin cancer.However, clinical trials were mainly confined to testing the efficacy of EGFR signaling inhibitors in regressing cSCCs. Studies that report a positive correlation between EGFR over-expression and invasiveness of cSCC reinforce the idea of targeting these proteins. However, the response rate for these interventions in clinical setting, especially with small molecule inhibitors, is low. This therapeutic resistance could be attributed to the possibility that multiple receptor tyrosine kinases over-expressed in cSCC leads to the activation of the same downstream targets causing decreased response to these drugs. Considering this hurdle, it would be interesting to assess the efficacy of drug combinations that targets receptor tyrosine kinase and its down-stream signaling. Proposing an effective drug combination requires evaluation of the phosphoproteome of tumor from cSCC patients to understand the activation profile of the tumor proteome. Of interest, some natural products, viz., curcumin, Quercetin, Kaempferol, EGCG, etc., directly binds and inhibits multiple proteins that operate downstream to RTK signaling. The efficacy of these phytochemicals can be leveraged to bring in more therapeutic effect. Some of these molecules exhibit poor pharmacokinetic properties, and hence drug delivery systems must be optimized to improve their bioavailability and retention time. These molecules should be tested for their efficacy to act alone or in combination with targeted therapies for better treatment outcomes. Encouraging evidence on this line is the effectiveness of cetuximab-curcumin phospholipid combination in treating cSCC. Similarly, we have reported the effectiveness of nanoformulations of curcumin in increasing its pharmacokinetic properties. Other than curcumin, multiple natural products are known to have anti-cancer effect against cSCC as assessed by preclinical studies. However, their inclusion in clinical trials requires rigorous preclinical testing of their effect on cSCC cells and animal models with different mutation status/signal aberration to bring in a more informed, personalized approach.

Most of the current preclinical studies using phytochemicals were focused on evaluating their efficacy as chemopreventives. To have a better understanding about their therapeutic potential, *in vivo* models of advanced cSCC should be used. PDX models, especially tissue recombinant models comprising cancer cells and tumor-associated stromal cells can be used for testing the efficacy of prospective drugs. A recent study by Hsu et al., has used a PDX-model of cSCC for the first time to study the anti-cancer effect of eribulin. At present, this is the only studythat has reported the use of PDX-model to study cSCC.[87] The use of

tissue recombinant models could be considered to better mimic human cSCC in mice. Sasaki et al., have categorized cancer-associated fibroblasts (CAFs) from cSCC into 3 subgroups based on the expression of CAF and epithelial to mesenchymal transition (EMT) markers, and these subgroups were correlated with different skin tumor phenotypes cSCC, Basal cell carcinoma (BCC), and malignant melanoma (MM).[118] This information could be used to construct tissue recombinant models wherein tumor cells are combined with CAFs.The inclusion of biomarkers of cSCC progression in assessing response to drugs can be a way forward in cSCC management. Two separate studies that used microarray datasets of cSCC reported that *EGR3, MYBL2* and *TK1* can be used as markers of cSCC progression.[119,120] Furthermore, the genomic expression/mutation analysis of tumors from treatment-responsive/non-responsive patients must be studied to understand determinants of therapeutic success. These can be used as biomarkers to predict therapeutic success of chemotherapeutic agents.

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AUTHOR CONTRIBUTIONS

M.S.G: Writing – original draft, Writing – review and editing. S.U.A.: Writing – review and editing. C.K.K. and T.P.R.: Writing – review and editing. R.J.A.: Conceptualization,Writing – review and editing, Supervision.

DECLARATION OF INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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