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Epigenetic regulation of RTK signaling

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Abstract

Receptor tyrosine kinase (RTK) signaling cascades coordinate intracellular signaling in response to growth factors, chemokines and other extracellular stimuli to control fundamental biological processes such as cellular proliferation, metabolism, and survival. Hyperactivation of pathways associated with growth factor signaling (e.g., RTK and downstream effectors including Ras, PI3K/AKT, Raf, and others) is a frequent event in human cancers, which uncouples ligand-mediated activation with signal transduction. While the contributions of direct genomic events are well understood as causative agents of hyperactive signal transduction, other non-heritable genomic modifications promote the activation of growth-factor associated signaling cascades. In this review we highlight epigenomic mechanisms by which hyperactivation of RTK-associated signaling cascades occur and may contribute to cancer.

Keywords

Cancer; Epigenetics; Receptor tyrosine kinases; signal transduction

Introduction

In the past few years, next-generation sequencing highlighted the importance of genomic alterations in cancer development – predominantly through mutational activation or amplification of oncogenes or functional inactivation of tumor suppressor genes. Unexpectedly, this research unveiled the high frequency of genomic alteration of chromatin readers, writers, and erasers in cancer, suggesting the importance of epigenomic regulation in the development of many cancer subtypes [1-3]. While it is well-understood that hyperactivation of receptor tyrosine kinase (RTK) and growth-factor associated signaling pathways is a frequent event in cancer, only recently has research implicated epigenomic mechanisms by which these pathways might become hyperactivated. In this review we discuss epigenomic mechanisms by which RTK-associated signaling is activated in cancer, and relevant therapeutic implications that these findings may have in the treatment of RTK pathway-activated cancers.

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RTK/Growth factor signal transduction

Receptor tyrosine kinase-regulated cellular signaling pathways coordinate integral cellular processes including proliferation, survival, metabolism, and growth in response to growth factors and chemokines. Approximately 60 receptor tyrosine kinases are encoded by the human genome [4] and are sensitive to extracellular levels of growth factors, chemokines, or other ligands, including but not limited to those belonging to the insulin, ERBB(HER/EGF), PDGF, HGF, VEGF, FDGF, and EPH receptor families. Of note, detailed reviews on these receptor families and the signal transduction they initiate have been published elsewhere [5-7]. In brief, intracellular signal transduction originates with ligand binding to the RTK, promoting receptor dimerization and tyrosine autophosphorylation within the RTK intracellular domains. RTK phosphorylation stimulates the association of effector proteins and adaptor proteins bringing still more effectors, propagating signal transduction. Downstream effector signaling networks that are frequently implicated in RTK-mediated oncogenic growth include the PI3K/AKT/mTOR, Ras/RAF/MAPK, FAK, JAK/STAT, and other pathways, also reviewed elsewhere [8-11]. Of note, hyperactivation of these downstream effector signaling networks promotes cellular growth, proliferation, migration, survival, metabolism, and other biological processes that are frequently associated with transformation and oncogenesis.

HDAC-mediated RTK effector expression and activity

Epigenetic alteration commonly occurs in cancers, and can affect RTK-mediated signal transduction and, as is more widely appreciated, gene transcription. Disruption of genome-wide histone acetylation has been identified as a frequent occurrence in cancer [12]. High levels of histone acetylation correlate with active gene transcription often in genes driving tumor growth and survival pathways, and inhibition or genetic ablation of histone deacetylases (HDACs) prevents oncogenic growth in some models of cancer [13]. While, in some cases, the mechanisms may not be entirely characterized, HDACs also have been found to directly or indirectly modulate RTK signal transduction, by regulating RTK effector gene expression and subsequent downstream signaling. Obviously other genes are regulated as well. The discovery of the connections between HDAC activity and cancer have initiated the development of small molecule inhibitors with activity against HDACs [14], and the clinical testing of combined HDAC and RTK pathway inhibition are in progress. Several relevant examples are discussed below (Figure 1).

HDAC inhibitors have been shown to induce the expression of pro-apoptotic transcription factor FOXO1, which is normally inactivated by AKT-mediated phosphorylation, in a mouse model of MYC-amplified, p53 mutant medulloblastoma [15]. Ectopic expression of FOXO1 in cells derived from these tumors and in human MYC-amplified medulloblastoma cell lines is sufficient to reduce cell viability and extend animal lifespan in allograft and xenograft models [15]. Importantly, combined HDAC and PI3K inhibition synergizes in models of MYC-amplified medulloblastoma *in vivo*; HDAC inhibitors increase FOXO1 expression, and PI3K inhibitors prevent AKT-mediated FOXO1 phosphorylation and nuclear exclusion such that FOXO1 can associate with chromatin and promote pro-apoptotic gene transcription [15]. The success of combined HDAC and PI3K inhibition in preclinical

studies launched the development of a dual HDAC/PI3K inhibitor, CUDC-907, which has preclinical activity in a variety of patient derived xenograft models of MYC-amplified solid cancers [16].

The success of combining HDAC inhibition with PI3K pathway inhibitors to synergistically inhibit oncogenic growth also extends to preclinical breast cancer studies. Combined mTORC1/2 and HDAC inhibition via MLN-0128 and trichostatin A (TSA), respectively, reduces AKT phosphorylation and prevents cap-dependent translation by limiting polysome assembly in a panel of transformed human breast cancer cell lines[17]. Because HDAC inhibition alone reduces AKT phosphorylation in the breast cancer cell lines tested, it is likely that HDACs modulate PI3K effector signaling through mechanisms independent and/or upstream of FOXO1 expression. Combined mTORC1/2 and HDAC inhibition also enhanced apoptosis, but it is unclear whether this is mediated by FOXO1 in breast cancer cell lines as is the case in the MYC-amplified medulloblastoma cell lines.

The development of a chromatin-mediated, reversible drug-tolerant state through the development of cancer stem cells in lung cancer cell lines has been shown to require either the histone H3 lysine 4 (H3K4) histone demethylase KDM5A or HDAC activity, and is dependent on IGF-1R signaling. In the EGFR-mutant, erlotinib sensitive PC9 lung cancer cell line, approximately 0.3% of cells develop a quiescent, drug tolerant persister (DTP) phenotype, and emergence of these cancer stem cells is dependent on the histone demethylase KDM5A[18]. KDM5A has been shown to complex with HDACs, and the yeast ortholog of KDM5A has histone deacetylase activity. Interestingly, the authors found that HDAC inhibition was sufficient to suppress the emergence of these DTPs. DTP formation also required the activation of IGF-1R signaling, which was mediated by KDM5A[18]. It is, however, unknown whether HDAC activity is also capable of activating IGF-1R signaling in this context. Exogenous IGF application promoted DTP emergence which was reversed following loss of KDM5A expression. Together these data suggest that KDM5A or, either directly or indirectly, HDAC activity enhances IGF-1R signaling and the development of a cancer stem cell-like population, which mediates acquired resistance to tyrosine kinase inhibitors. This research has encouraged the preclinical development and testing of KDM5-family inhibitors to minimize the emergence of drug tolerant cells and subsequent acquired resistance[19, 20].

HDAC inhibition has also been linked to modulation of MAPK effector gene expression. KEGG pathway analysis of cells treated with the HDAC inhibitor TSA demonstrated that HDAC inhibition reduces RTK/MAPK effector expression. As a result, HDAC inhibition correlated with reduced ERK phosphorylation[21]. These studies were performed in normal cardiac myotubes and should be repeated in *in vitro* and *in vivo* cancer models characterized by high RTK/Ras/MAPK activity to determine whether HDAC inhibition correlates with anti-proliferative properties linked to the repression of this pathway.

As a result of the success of HDAC inhibitors in preclinical and clinical studies of solid and hematological malignancies, the HDAC inhibitors Vorinostat, Rhomidepsin, Panobinostat and Belinostat are now FDA approved in the treatment of some myelomas and leukemias[22].

Numerous early stage clinical trials are underway, testing the efficacy of combined HDAC and RTK-pathway inhibitors (e.g., mTOR, EGFR) in the treatment of cancer.

RTK acetylation as a mechanism to modulate RTK activity

Direct acetylation of RTKs also modulates the activity of RTKs including EGFR and VEGFR. EGFR has been shown to be acetylated on K684, K836 and K843 by the acetyltransferase CBP in breast cancer cell lines[23]. Moreover, EGFR acetylation and phosphorylation was enhanced following HDAC inhibition via SAHA, suggesting that HDAC inhibition may promote EGFR activation[23]. Because EGFR acetylation primes EGFR for subsequent phosphorylation and activation, it is tempting to speculate that the FDA approved and clinically utilized HDAC inhibitors described above may promote accumulation of phosphorylated and activated EGFR. It is possible that EGFR-mediated acetylation is one mechanism of acquired resistance to HDAC inhibitors, although further research is necessary to test this hypothesis. Similarly, VEGFR acetylation on four residues by p300 has been shown to promote a favorable structural conformation such that it is subsequently phosphorylated and activated, although it is unknown whether VEGF acetylation is relevant in the context of HDAC inhibitor-mediated resistance[24].

DNA hypermethylation and tumor suppressor gene silencing

A high frequency of CpG dinucleotides, referred to as CpG islands, is commonly found at or near gene promoters, and is subject to DNA methylation [25]. CpG hypermethylation typically occurs at the promoters of genes required for development, lineage specificity, as well as endogenous retroviral sequences and proto-oncogenes in order to suppress DNA transcription[26]. However, aberrant CpG hypermethylation is also associated with cancer. Specific hypermethylation of CpG islands found within promoters of tumor suppressor genes is associated with gene silencing and transcriptional repression[27]. While genomic alterations that functionally inactivate the PIP₃ lipid phosphatase and tumor suppressor PTEN are frequently observed in cancer, PTEN expression is also silenced epigenetically through promoter hypermethylation[28]. Early studies demonstrated that PTEN mutations account for a large percentage of cases in which PTEN is functionally inactivated in tumors. However, loss of PTEN expression also was found to occur in prostate cancer xenografts characterized by an intact PTEN genomic locus. PTEN expression could be restored with the treatment of the DNA demethylating agent 5-Azadeoxycytidine (azacitidine), suggesting epigenetic mechanisms of PTEN regulation [29]. PTEN promoter hypermethylation was also observed in approximately 39% (26 of 66 cases) of human gastric carcinomas, of which 73% (19 of 26) of the cases correlated with a loss of PTEN expression by immunohistochemistry [28]. Later studies demonstrated PTEN promoter hypermethylation in other cancers including soft tissue sarcomas, which is especially important due to the low frequency with which PTEN genomic alterations occur in this cancer subtype[30].

Because PTEN promoter hypermethylation has not been found in all cancer subtypes, it is likely that this process occurs in a context dependent manner. It is tempting to speculate that patients with tumors characterized by PTEN epigenetic suppression maybe candidates for

PI3K p110 β isoform-selective inhibition alone or in combination with DNA methyltransferase inhibitors decitabine or azacitidine. This is the result of previous work demonstrating that tumors from mouse models characterized by PTEN genetic loss or functional inactivation are primarily sensitive to p110 β , but not p110 α , inhibition [31, 32].

DNA hypermethylation of genes encoding hormone receptors as a mechanism of endocrine therapy resistance

Loss of hormone receptor expression [estrogen receptor (ER), progesterone receptor (PR), and androgen receptor (AR)] in breast and prostate cancers is an important mechanism of endocrine therapy resistance. In some cases endocrine therapy resistance is linked to the epigenetic repression through promoter hypermethylation of the implicated hormone receptor. ER-negative breast cancer cell lines grown in the absence of estrogen were found to have reduced ER mRNA expression, elevated capacity for DNA methylation, and specific DNA CpG dinucleotide hypermethylation of the ER promoter [33]. This ER promoter hypermethylation was sensitive to DNA demethylating agents, which promoted ER re-expression. Further work demonstrated that ER-negative and PR-negative primary human breast cancer tissues and metastatic lesions are frequently characterized by hypermethylation of the ER and PR promoters, respectively [34]. DNA hypermethylation of the AR has also been shown to contribute to endocrine therapy resistance in prostate cancer. Similar to ER-negative breast cancer cell lines, AR-negative prostate cancer cell lines are frequently characterized by AR promoter DNA hypermethylation, which can be reversed following treatment with the DNA demethylating agent azacitidine [35, 36]. These data suggest that hormone therapy resistant breast and prostate cancers may benefit from the therapeutic treatment with DNA demethylating agents such as azacitidine or decitabine.

DNA hypermethylation indirectly promotes the interaction of constitutive enhancers with oncogenic loci

DNA hypermethylation has also been shown to indirectly modulate RTK signaling in the context of IDH1 mutant glioblastomas. An oncogenic metabolite, 2-hydroxyglutarate (2-HG), is produced by the IDH1^{R132H} mutant enzyme that inhibits DNA demethylation [37]. The resulting hypermethylation of cohesion and CCCTC-binding factor (CTCF)-binding sites prevents the association of the insulator protein CTCF with chromatin. The lack of CTCF binding disrupts the normal topological domains or neighborhoods (discrete looping structures separated by insulators such as CTCF) in chromatin, which can promote gene expression should a constitutive enhancer interact with an oncogenic locus. DNA hypermethylation as a result of IDH1-mutant expression has been shown to result in the loss of insulator-associated repression of the RTK PDGFRA, enhancing its expression in IDH1 mutant gliomas [38]. Treatment with DNA demethylating agents reversed this effect, reducing PDGFRA expression and slowing oncogenic growth [38]. Because IDH1 (or IDH2) mutation and 2-HG production occurs in acute myeloid leukemia, cholangiocarcinoma, and other cancer types, it is possible that aberrant PDGFRA expression also contributes to oncogenic growth in these contexts.

Importantly, mutation of CTCF insulator binding sites occurs across cancer types [39] and disruption of CTCF insulator neighborhood boundaries enhances proto-oncogene and oncogene expression and activation independently of IDH1 mutation[40]. Surveying more than 50 cancer types with 2300 whole genome sequencing samples that collectively contained more than 13 million unique somatic mutations, one study identified an enrichment of CTCF binding site mutations specifically at chromatin neighborhood boundaries [40]. As a result of CTCF binding mutations, the RTK and proto-oncogene FGFR1 is aberrantly expressed in hepatocellular carcinoma (HCC), while in esophageal cancer expression of the RTK effector and proto-oncogene BRAF is elevated [40]. These data suggest that HCC tumors characterized by elevated FGFR1 expression may benefit from FGFR inhibitors, which are currently in clinical development for the treatment of HCC [41]. While isoform-selective FGFR inhibitors are in preclinical development at this time, because FGFR1 is not abundantly expressed in the normal liver, FGF1R-selective inhibition may offer superior on-target inhibition with minimal side effects in HCCs with high FGFR1 expression. It is, however, unknown whether reduction of BRAF expression or BRAF inhibition in esophageal cancers characterized by aberrant wild type BRAF expression reduces oncogenic growth. It is tempting to speculate that cancers characterized by DNA hypermethylation or mutation of insulator binding sites may generally reduce association of insulators with chromatin and disrupt neighborhood regulatory structures, resulting in aberrant oncogene expression.

DNA hypomethylation of RTK signaling-associated oncogenes

While global DNA hypermethylation is frequently observed in cancer, localized promoter hypomethylation of oncogenic loci also occurs, promoting their aberrant expression. Several studies have identified oncogene hypomethylation that mediate RTK signal transduction in cancer. More than thirty years ago, H-ras and/or K-ras promoter hypomethylation was observed exclusively in primary human colon adenocarcinomas, primary human small cell lung carcinomas, and metastatic lesions, demonstrating for the first time that oncogene methylation status may be lost in human cancers [42]. R-ras promoter hypomethylation was also observed in gastric cancer [43]. When Ras is hypomethylated and its expression is increased, signaling of its potent oncogenic effectors PI3K and MAPK is enhanced. Indeed, first-generation HDAC inhibitors restored Hras promoter methylation, reduced H-ras expression, and prevented cell growth in colon and gastric cancer cell lines characterized by hypomethylated H-ras[44].

Hypomethylation of RTK-associated oncogenes has also been implicated in resistance to targeted therapies. While BRAF inhibitors are associated with high objective response rates in patients with BRAF-mutant melanomas, drug resistance and subsequent tumor recurrence can develop. Compensatory BRAF mutation, PTEN inactivation, hyperactivation of compensatory RTK signaling pathways, and oncogene overexpression, amongst others, confer resistance to BRAF inhibition in BRAF-mutant cancers[45-47]. Upregulation of EGFR expression was recently shown to mediate acquired BRAF inhibitor resistance in melanoma cell lines and in human BRAF-mutant melanoma patient samples following treatment with the BRAF inhibitors dabrafenib or vemurafenib[48]. EGFR overexpression was linked to EGFR promoter DNA hypomethylation[48]. Evaluation of genome-wide DNA

methylation demonstrated that EGFR expression is also upregulated through DNA hypomethylation of upstream and downstream EGFR enhancers [48]. Moreover, BRAF-inhibitor resistant melanoma cell lines were resensitized by dual EGFR and BRAF inhibition. The mechanism by which EGFR promoter and enhancer hypomethylation occurs is, however, unknown. These data suggest that DNA hypomethylation upregulates EGFR expression, which can mediate acquired inhibitor resistance in BRAF-mutant melanoma cell lines. Combined EGFR and BRAF inhibition may minimize tumor relapse due to the prevention of acquired resistance from BRAF-inhibitor monotherapy.

RTK-signaling has also been shown to regulate genomic loci-specific DNA methylation status as part of a feed-forward loop that enhances growth factor-associated RTK signal transduction. Concurrent genetic deletion of the AKT targets GSK3 α/β in mouse embryonic fibroblasts was reported to reduce expression of the DNA methyltransferase DNMT3. A reduction in DNMT3 expression promoted DNA hypomethylation specifically at the regulatory regions of the IGF2/IGF2R imprinted loci[49], enhancing expression of IGF2 and IGF2R [50]. PI3K-mediated AKT activation was sufficient to enhance IGF2/IGF2R promoter hypomethylation. These data indicate reactivation of IGF2/IGF2R through promoter hypomethylation may function as a mechanism of feed-forward RTK pathway activation [50]. Notably, hypomethylation of the imprinted gene IGF2 correlates with a poor clinical outcome in colorectal and esophageal cancers, suggesting the importance in maintaining RTK loci-specific imprinting in cancer[51, 52].

While some of the above indicated studies do not elucidate the mechanism(s) by which specific genomic loci are hypomethylated, it should be noted that general inactivation of DNA methyltransferase activity can result in DNA hypomethylation and oncogenic growth. For example, nonspecific DNA hypomethylation is associated with tumor formation in genetic mouse models with reduced DNA methyltransferase 3 (DNMT3) activity [53]. It is likely that the separate or combined function of DNA methyltransferase genes DNMT1/3a/3b controls both general and specific, context dependent, DNA methylation in cancer [54].

Conclusion

The impact of hyperactive growth factor-associated RTK signaling on cancer development, metastasis, and resistance makes kinase nodes within these pathways attractive pharmaceutical targets. However, effectively suppressing RTK-associated signal transduction in cancers may depend on targeting not only RTK and RTK effector activity but also the epigenetic enzymes that control expression and activity of RTKs and their effectors. The combination of these two classes of enzyme inhibitors for the treatment of tumors featuring RTK pathway-activation may greatly improve clinical outcome.

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Figure 1. Chromatin-mediated changes to RTK effector expression, activity, and phosphorylation

HDAC inhibition has identified multiple mechanisms by which RTK effector activity or expression is suppressed. Left: HDAC inhibition induces the expression of the pro-apoptotic transcription factor FOXO in MYC-amplified cancers. HDAC inhibition reduces phosphorylation of the RTK/PI3K effector AKT in preclinical breast cancer studies. HDAC and mTORC1/2 inhibition in this context durably inhibits AKT phosphorylation and polysome formation which is required for efficient cap-dependent translation. HDAC inhibition reduces phosphorylation of the RTK/MAPK effector ERK in normal heart tissue. Right: The emergence of a drug-tolerant cell population is dependent on either HDAC or KDM5A activity; HDAC inhibition or KDM5A ablation inhibits the formation of drug-tolerant cells. This chromatin-mediated effect activates IGF-1R and is blocked by loss of KDM5A expression. Acetylation of EGFR and VEGFR promotes RTK phosphorylation and activation. These preclinical data are suggestive of a possible mechanism of acquired resistance to HDAC inhibitors.

Figure 2. Methylation-associated regulation of RTK signaling

(A) CpG dinucleotide Hypermethylation of the PTEN promoter functions to suppress the PTEN tumor suppressor expression and activity, activating PI3K effector signaling. (B) Endocrine therapy resistance in breast and prostate cancers have been linked to the hypermethylation of CpG dinucleotides within the ER, PR, and AR promoters. Hypermethylation silences these hormone receptors, promoting resistance to associated hormone therapies. (C) Promoter hypomethylation of Ras and other RTK effectors enhance oncogene expression and may contribute to oncogenic growth. (D) Aberrant DNA hypermethylation inhibits the association of DNA-methylation sensitive insulators chromatin and maintenance of distinct chromatin domain or neighborhood structures (Top). The subsequent association of enhancers or superenhancers with proto-oncogene or oncogene promoters (e.g., PDGFRA) then promotes the expression of RTKs that are known oncogenic drivers. The disruption of chromatin domain or neighborhood structures can also occur through DNA methylation independent mechanisms, such as mutation of CTCF insulator binding motifs (Bottom).