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Protein Kinase Signalling Networks in Cancer

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Summary

Protein kinases orchestrate the activation of signalling cascades in response to extracellular and intracellular stimuli to control cell growth, proliferation, and survival. The complexity of the numerous intracellular signalling pathways is highlighted by the number of kinases encoded by the human genome (539) and the plethora of phosphorylation sites identified in phosphoproteomic studies. Perturbation of these signalling networks by mutations or abnormal protein expression underlies the cause of many diseases including cancer. Recent RNAi screens and cancer genomic sequencing studies have revealed that many more kinases than anticipated contribute to tumorigenesis and are potential targets for inhibitor drug development intervention. This review will highlight recent insights into known pathways essential for tumorigenesis and discuss exciting new pathways for therapeutic intervention.

Introduction

The discovery that the Philadelphia chromosome generates the constitutively activated BCR-ABL oncogenic tyrosine kinase and drives tumorigenesis of chronic myelogenous leukemia was paramount to the development of the small molecule inhibitor imatinib (Gleevec^{TM}) [1]. This success has spawned major efforts by both academic and pharmaceutical researchers to identify aberrantly regulated signalling pathways and develop novel small molecule compounds targeting these networks in cancer. For example, promising results from a phase 1 clinical trial, followed by an extended phase, with a novel B-RAF inhibitor, PLX4032, that targets V600E mutant B-RAF (discussed in detail below), suggest that therapy could benefit greater than 60% of melanoma patients harboring an activating B-RAF mutation [2,3]. Indeed, these data are compelling and should only intensify our search for cancer-associated kinases and the development of novel compounds targeting these kinases.

Identification of essential cancer kinases or driver mutations in kinases is accelerating at a rapid pace due to large scale RNAi screens and cancer genomic sequencing efforts [4–12]. Filtration of these data to identify viable targets in cancer patients is essential as we progress into the age of personalized medicine. These cancer-associated kinase targets will require detailed characterization of their specific roles in the process of tumorigenesis, and investigation of whether inhibition or activation of the pathway in which they are involved will promote cancer cell death and tumor regression. To this end, we will need to

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characterize the nonsynonymous mutations identified in kinases to identify amino acid changes that alter the kinase in such a manner that it has either greater or reduced catalytic activity or otherwise altered function. Such gain-of-function (GOF) or loss-of-function (LOF) mutations will likely contribute to the initiation and/or progression of cancer [7,9,13–18]. Many examples of activating mutations in kinases are known (for example PIK3CA, discussed below), but cancer kinome sequencing has also begun to uncover mutations that cause loss of function. For example, mutations in c-Fes reported in colorectal carcinoma [19] and cancer mutations identified in MAP2K4 decrease the catalytic activity, suggesting that the normal function of c-Fes and MAP2K4 is to suppress abnormal proliferation or growth [14,19]. In a complementary approach, RNAi screens are identifying essential kinases whose activity is required in oncogene-induced tumor formation [20–22]. This is exemplified by recent studies using RNAi libraries to identify kinases essential for K-RAS-mediated oncogenesis [20,21], which have pinpointed promising new targets for cancer patients harboring activating mutations in K-RAS, including the STK33, TBK1, and PLK1 kinases. Of these kinases, STK33 is largely uncharacterized, but has also been suggested to harbor a driver mutation in pancreatic cancer, making it a potentially exciting new target [23]. To verify the importance of a kinase in tumor progression or maintenance, it is important to integrate data from RNAi screens, kinase inhibitor screens, and cancer genomic studies [8,24], to pinpoint pathways where activation or inactivation is essential for tumorigenesis [14].

In addition to identifying kinases essential for specific oncogene-induced tumors, global kinome-wide RNAi screens in tumor cell lines have also uncovered many understudied or novel kinases essential for tumor cell survival, even though these kinases are not necessarily mutated [5,6,10–12,25]. Interestingly, there is minimal overlap between essential kinases required for survival of different tumor cell lines. For two well-studied cell lines (HeLa and 293T) overlap was 25%, but surprisingly for 4 different nonsmall cell lung cancer (NSCLC) cell lines it was only 5% [5]. Importantly, these studies also identified STK33 as an essential kinase, and in addition other novel kinases with potential driver mutations, such as SgK495 [5,6,10–12]. These studies shed light on the striking diversity among cancer cells derived from the same cell of origin and highlight the unique evolutionary path a cancer cell can take on the road to a malignant phenotype, and underscores the need for better understanding of the role that novel and understudied kinases play in cancer.

PIKing targets AKTurately

The importance of the PI3K/Akt pathway in cancer was first established by identification of the tumor-suppressor phosphatase PTEN, which dephosphorylates the 3'-position of the inositide ring to eliminate the lipid second messenger PIP₃ and terminate signalling through this pathway [26,27]. Firmly establishing the activation of this pathway as an essential node in tumorigenesis was the discovery of activating mutations in *PIK3CA* [15], the gene encoding the p110 α PI3K catalytic subunit, which result in constitutive activation of this pathway [28]. In addition, mutations in other upstream activators, such as EGF receptor (EGFR) and K-RAS, and AKT1 itself can drive activation of this pathway in many different cancers [29]. Recent studies have highlighted new ways for cancer cells to promote signalling through this pathway [30], and it is estimated activation of this pathway is likely to be crucial for a majority of cancers (upwards of 70% of primary breast tumors, for example) [31]. Mutations in various components of this pathway such as LOF mutations in PTEN, GOF mutations in PI3K, or GOF mutations in AKT itself all result in the common endpoint of increased AKT activation suggesting that AKT inhibitors could be used to treat patients with mutations in any of these respective enzymes. However, it is possible that patients with mutations in AKT1 will respond better to an AKT1 specific inhibitor, and patients with PI3K mutations will respond better to a PI3K specific inhibitor.

One novel mechanism that decreases PTEN expression involves the loss of expression of the pseudogene PTENP1, whose transcripts can act as a decoy for microRNAs targeting PTEN mRNA. Loss of PTENP1 leads to increased PTEN microRNA targeting and decreased PTEN expression, and subsequently leads to the activation of the PI3K/Akt pathway. In consequence, it is not surprising that the *PTENP1* gene is lost in various cancers (Figure 1) [30].

Downstream of PI3K is its primary target, AKT, which requires the lipid second messenger PIP₃ for activation [24]. Binding of PIP₃ to AKT's PH domain allows constitutively bound PDK-1 to phosphorylate AKT at the activation loop site, Thr308 (Akt1) [32]. AKT is also phosphorylated at the hydrophobic motif, Ser473 (AKT1), by the mTORC2 kinase complex, which enhances AKT activation and its subsequent phosphorylation of downstream substrates, especially, Forkhead Box O (FOXO) family of transcription factors [33,34]. The mTORC2 kinase complex can interact with DEPTOR, which suppress the activity of this kinase complex and decreases hydrophobic motif phosphorylation of Akt [35]. Depletion of DEPTOR results in an increase in Akt phosphorylation (and an increase in S6K phosphorylation because DEPTOR also inhibits mTORC1) and loss of DEPTOR is a common event in multiple myelomas [35]. Consistent with the importance of increased Ser473 phosphorylation in tumorigenesis is the observation that the PHLPP phosphatases have decreased expression or activity in colon, prostate, breast, and pancreatic cancers, as well as CML [36–40]. These phosphatases modulate the amplitude of AKT signalling by regulating phosphorylation of specific Akt isoforms at Ser473 [41,42]. Additional posttranslational modifications of AKT have emerged as another quality control mechanism to regulate its activation. Specifically, TRAF6 promotes polyubiquitylation of AKT, generating Lys63-branched chains, which enhance membrane recruitment of AKT and subsequent activation (polyubiquitylation also contributes to hyperactivation of the cancer mutant Akt – E17K) [43]. Consistent with the role of TRAF6 in promoting the activation of Akt, mice lacking TRAF6 have decreased tumorigenic potential [43]. The existence of these multifaceted quality control mechanisms that ensure proper regulation of the PI3K/Akt pathway provide multiple sites for aberrant regulation of this pathway and indicate that tight regulation of the pathway is essential to prevent inappropriate proliferation, survival, and cell growth (Figure 1). These factors must be taken into consideration when targeting various components of this pathway, since feedback loops are used to temper signalling through this pathway [44–46]. Therefore, inhibitors of the mTORC1 complex, such as rapamycin, can have the unintended consequence of activating the PI3K/Akt pathway, and may need to be used in combination with other inhibitors, such as a PI-3 kinase inhibitor, or a more promiscuous inhibitor that would target both PI3K and mTOR [47,48].

Successful Targeting of Mutant B-RAF

Mutations in B-RAF (particularly V600E) have conclusively been characterized as driver mutations that promote the constitutive activation of the MEK1/2-ERK1/2 signalling pathway, which activates transcription to drive cellular proliferation [49]. Activating B-RAF mutations are frequent events in melanoma; however, inactivating mutations have also been identified [49,50]. Attempts to develop MEK1/2 inhibitors for clinical use have so far been unsuccessful [51]; however, mutant B-RAF is proving to be a druggable target, and newly developed inhibitors are showing therapeutic efficacy in clinical trials for what was once an unresponsive cancer (metastatic melanoma) [2,3]. However, there are issues arising with B-RAF inhibitors, particularly related to drug resistance and activation of c-RAF, which can have the unintended consequence of activating the RAF-MEK-ERK pathway [52–54]. Recent findings have highlighted a unique mechanism where ATP-analogue RAF inhibitors bind to either B-RAF or c-RAF and induce a conformational change that is conducive to dimer formation (homodimer c-RAF/c-RAF) or heterodimer (B-RAF/c-RAF) [52–54]. The

drug-free c-RAF molecule in the dimer is then transactivated to promote activation of the MEK-ERK pathway. These effects are dependent upon mutationally activated K-RAS, and the inhibited RAF molecule (either genetically or chemically inactivated) acts as a scaffold to promote signalling from mutant K-RAS resulting in activation of the MEK-ERK pathway, which is mediated by the drug-free c-RAF molecule present in the dimer [52–54]. Combined these studies illuminate a complex mechanism where inactivation of RAF (either chemically or mutationally) can ultimately lead to activation of the RAF-MEK-ERK pathway (Figure 2). Therefore, patient mutation status should be taken into consideration when treating melanoma patients, since B-RAF inhibitors can activate this signalling pathway in patients with RAS mutations [52–54]. Additionally, greater than 80% ERK inhibition is required for clinical efficacy and this should be taken into consideration when monitoring effectiveness of pathway inhibitors [3]. Lastly, these studies suggest that mutations in RAS may be a mechanism to promote resistance to RAF inhibitors.

Eph Receptors in Cancer

The importance of ephrin-Eph receptor interactions and signalling downstream of this large family of receptor tyrosine kinases (RTKs) was established immediately upon the discovery of the first Eph receptor (EphA1) [55]. The role of Eph receptor signalling in cancer has largely been inferred based on the effects of overexpression or reduced expression suggesting oncogenic or tumor suppressive roles, respectively [56]. The recent identification of Eph receptor mutations, particularly in lung cancer, further implicates this family of RTKs in tumorigenesis [4,14]. The possible roles of Eph/ephrin signalling in cancer are complex and one could readily postulate a number of tumorigenic mechanisms. The promiscuous ability of EphA and EphB receptors to interact with multiple ephrin-A and ephrin-B ligands, respectively, combined with the fact that both ephrin-A and ephrin-B ligands are membrane-anchored and presented to Eph receptors on the surface of neighboring cells, which can themselves receive reverse signals upon binding Eph receptors, adds further complexity. For example, EphB2 is implicated as tumor suppressor in colon cancer in mice (as well as EphB3/4), and this is likely dependent upon kinase-independent functions that regulate migration through p110 α PI-3 kinase. However, there are tumor-promoting functions of the EphB2 receptor that are dependent upon kinase activity and are mediated through Abl kinase and cyclin D1 [57]. It is likely that EphB2 tumor promoting activity is required for initial stages of tumorigenesis, but dispensable for tumor progression. Furthermore, loss of EphB2 kinase-independent functions that suppress migration are likely required for tumor progression [57]. Since the cancer mutations in EphA3 appear to be inactivating, another potential mechanism would be through LOF mutant Eph receptors; these could block activation of other Eph receptors through coclustering or by sequestering ephrin ligands, and, like other kinase-dead mutants, act in a dominant-negative fashion to suppress negative feedback signalling to downstream targets such as AKT and ERK, which should result in increased signalling through these oncogenic pathways [56].

Novel Cancer-Kinase Pathways

As cancer genomic screens continue to expand our understanding of the landscape of somatic mutations in cancer, novel cancer-associated kinases will emerge as biomarkers and possible targets for intervention. Recently, cancer-associated mutations in MAP2K4 (MKK4/JNKK1) were shown to be loss of function, promote transforming activity, possibly as a result of acting in a dominant-negative manner to suppress the function of the WT allele [14]. This suggests that decreased signalling through the pro-apoptotic JNK pathway can promote tumorigenesis, and that other candidates mutated in cancer whose loss of function will contribute to decreased JNK pathway signalling (including possibly JNK2 itself, which is mutated at Arg162 in cancers of the large intestine) are likely to emerge. Consistent with

this, LOF mutations in PKC family members (such as the D294G mutation in PKC α in thyroid cancer [58–60]) could suppress signalling through this pathway through loss of PKC-mediated S129 phosphorylation, which stimulates signalling through the JNK pathway [61].

In addition to cancer genome sequencing, phosphoproteomic analyses will reveal novel signalling targets that are hyper- or hypophosphorylated in cancer and essential to tumorigenesis. For instance, a survey of tyrosine phosphorylation events in advanced colon carcinoma cells revealed that SgK223, a putative pseudokinase, is hyperphosphorylated on tyrosine in SRC-transformed cells and is required for Src-induced anchorage independent growth [62]. Similarly, Rikova et al. identified several tyrosine kinases with increased phosphorylation in lung cancer. Specifically, these studies revealed a novel link between the ALK, DDR1, and ROS kinases and NSCLC [63].

Elucidating novel signalling pathways utilized by more commonly mutated protein kinases should also expand the potential entry points for developing targeted therapies. For example, the well described tumor suppressor LKB1, which acquires loss-of-function mutations in a variety of cancers (for a thorough review see Shackelford et al. [64]), was recently shown to activate the NUA1 AMPK family member [65]. This activation leads to the phosphorylation of MYPT1 (myosin light chain phosphatase 1) and subsequent suppression of its phosphatase activity by binding to 14-3-3, ultimately leading to increased MLC2 phosphorylation [65]. Loss of function mutations in LKB1 could ultimately lead to increased MYPT1 activity (due to decreased phosphorylation by NUA1) resulting in a decrease in MLC2 phosphorylation. Loss of MLC2 phosphorylation could promote increased homotypic tumor cell adhesion, increased cellular aggregation, and provide a positive feedback loop for cancer cell proliferation. Additionally, this could provide a mechanism to suppress actomyosin contractility, which could maintain Rac1 in active state to promote mesenchymal-type movement (if actomyosin contractility is low, then we would expect decreased levels of ROCK1 activity resulting in decreased ARHGAP22 activity towards Rac1)[66] (Figure 3). Alternatively, MYPT1 can interact with ERM proteins (ezrin and moesin) as well as PLK-1 (polo-like kinase 1) and regulate the phosphorylation of these proteins, providing additional pathways downstream of MYPT1 that may be impacted by increased activation in cancer, and this possibility should be taken into consideration [67,68]. Consistent with the LKB1/NUA1/MYPT1 pathway being important in tumorigenesis, cancer kinome sequencing studies have revealed mutations in kinases that positively feed into this pathway that we would predict to be loss of function mutations, such as MLCK2, MRCK (CDC42BPA) and DAPK3. For DAPK3, we have observed that these mutations are indeed loss-of-function mutations (J.B. and T.H., unpublished observations) (Figure 3). However, the identification and characterization of activating mutations in ROCK1 suggest that increased MLC2 phosphorylation can also be important for cancer progression and migration, which is not surprising, and demonstrates the complexity and uniqueness of tumor evolution [69]. Discovery of such novel pathways, where mutations in several enzymes lead to a common output, should result in the development of novel cancer therapies targeting the key molecule and these therapies could be applicable to a wider range of tumors that find multiple ways to accomplish a similar phenotype.

Summary

The approach of tailoring treatments to individuals has the promise of turning cancer into a manageable disease. These new age therapies directed at the molecular aberrations present in a tumor, will have less side effects than traditional chemotherapeutics, and have the potential to transform deadly cancers into chronic diseases that can be managed. New

technologies are quickly accelerating our understanding of the landscape of enzymes that contribute to tumorigenesis and there is real promise in novel targets for larger pharmaceutical companies. Kinases mutated at a lower frequency (for example 3% in lung cancer) still should have a large patient cohort (greater than 6000 per year in the US) and could result in life saving therapies in greater than 4000 lung cancer patients per year. The goal will be to identify targets mutated at a lower frequency that are nonetheless still required for tumorigenic maintenance and will represent an Achilles heel in a subset of tumors. If we can begin to take these small steps to developing life saving therapies in small percentages of cancers, then eventually we can reach the goal of making all cancers a manageable disease.

Going forward new approaches must be developed to identify proteins that are mutated at a lower frequency yet are still crucial for tumorigenesis and thus harbor somatic driver mutations. Use of bioinformatic tools that predict the likelihood that a given mutation will alter the function of a kinase will be essential in pinpointing cancer-associated kinases [70–73]. Using these bioinformatic web-based applications, researchers will be able to predict which novel or understudied kinases pinpointed by kinome sequencing efforts possess almost exclusively driver mutations, and are likely to be altered functionally and contribute to tumorigenesis, and are therefore worthy of further study. The study of cancer-associated kinases has the potential to illuminate mechanisms of carcinogenesis, and lead to the discovery of new signalling pathways essential to tumorigenesis.

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- of special interest
- of outstanding interest

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Figure 1.

Mechanisms of activation of the PI3K/Akt pathway in cancer. The traditional mechanism for activation of the PI3K/Akt pathway is illustrated for normal cells, where growth factors bind to their receptors (e.g. EGFR) to promote their activation and phosphorylation of targets, such as Gab1, creating phosphotyrosine-binding sites for proteins that contain SH2 domains, such as the regulatory subunit of PI3K, p85. Subsequent activation of PI3K initiates signalling through the pathway. Termination of signalling is accomplished by direct dephosphorylation of Akt by PP2A and PHLPP and elimination of the lipid second messenger PIP₃ by the lipid phosphatase PTEN. Other mechanisms to suppress signalling through this pathway include DEPTOR and PTENP1. In cancer cells mutations in these kinases or phosphatases, coupled with decreased expression of negative regulators (DEPTOR and PHLPP) promotes hyper-activation of this pathway. Filled red shapes indicate negative regulators of the pathway.

Figure 2.
Targeting mutant B-RAF. Inhibitors specific for mutant B-RAF shut down signalling through the RAF-MEK-ERK pathway in cancer cells with activating mutations in B-RAF (V600E), but activate signalling through this pathway in cells with GOF RAS mutations.

Figure 3. Shedding light on a novel signalling pathway. The recent discovery that LKB1 can activate NUA1, leading to inactivation of MYPT1 and increased MLC2 phosphorylation, suggests this pathway may be important in cancers with LKB1 LOF mutations. In cancer LKB1 LOF mutations should result in activation of MYPT1, decreased MLC-2 phosphorylation, and increased tumor cell-cell adherence, which could positively feedback to promote proliferation. LOF mutations in other kinases, such as DAPK3, could be a means for a tumor cell to reach the same end point.