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METTL13 Mediated eEAF1A Methylation and RAS Driven Pathway in Various Cancers - A Potential Therapeutic Target?

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ABSTRACT

The incidence of cancers such as lung, pancreas, and bladder has increased in the last few decades. We urgently need to identify potent markers, which may help in prognosis and result in productive treatment strategies. In certain cancerous cell lines such as lung and pancreas, it has been observed that it undergoes dimethylation at lysine K55 on eEAF1A protein. How so ever, the modification of its functionality and the enzyme mediators are not yet known. Methyltransferase like Protein 13 (METTL13) catalyzes the methylation of eukaryotic elongation factor 1α (eEF1A) and increases the intrinsic GTPase activity of eEF1A in vitro and higher translational protein production in cells through RAS-driven pathway leading to progression of neoplastic growth. RAS pathway has been found to be involved in the regulation of various cancers which proves to be a promising area for future prognosis, targeted therapy and management, but their crucial aspects need to be elucidated more so that it merits further research.

INTRODUCTION

The vast majority of the proteins are believed to have one specific function. During the course of evolution, some proteins in order to meet the demands of the complex cellular milleu has acquired additional functions. However the eEF1 complex subunits have acquired such moonlight functions without alternative forms. eEF1A is a GTPase regulated gene which synthesises protein that is an essential component of translational machinery. eEF1A which is present as a GTP-bound form, binds and delivers aatRNAs to the A site of the ribosome. When a correct codon-anticodon pair is formed, hydrolysis of GTP takes place through a conformational change in the ribosome along with release of eEF1A-GDP. As the dissociation rate of GDP from eEF1A is very slow, GDP is actively exchanged with GTP by guanine nucleotide exchange factor (GEF) eEF1B in order for eEF1A to participate in another round of elongation [1].

A spotlight on the relationship between eEF1A and METTL13 gene in the upregulation of protein translation in many oncogene-driven tumors which is essential to sustain neoplastic growth has been lately noted [2]. METTL13 is a human gene located at 1q24.3 and was initially found from rat liver. Even though the eEF1A methylation sites have been known for decades it was only recently found by Liu, Hausmann and colleagues that methylation of eEF1A, a GTPase that is non ribosomal component of the translational machinery, is dimethylated at Lysine55 (eEF1AK55me2) by METTL13, leading to an increase in global protein synthesis in pancreatic, lung and bladder cancer cells [2].

It was also postulated that variations in the elements of translational apparatus, along with eEAF1A1/2 overexpression, might be a necessary tool to kick start oncogenic growth signalling pathways like RAS-MAPK, PI3K/AKT/mTOR in neoplastic cell proliferation, migration and invasion [3,4]. Lung and pancreatic cancer are the most common forms of cancer leading to mortality, in which Lung adenocarcinoma (LAC) the most frequent histological type and Pancreatic ductal adenocarcinoma (PDAC) the most prevalent pancreatic subtype. METTL13 expression were found to be overexpressed in vast majority of LAC and PDAC cases [5] through RAS-activated pathway (eg-PI3K), expressing oncogenic mutant KRAS [6,7]. Interestingly in a study published by Nature scientific reports showed downregulation of METTL13 expression in bladder carcinoma leading to cell proliferation, migration and invasion [8].

Many therapeutic targets were identified that hit multiple pathways such as PI3K/AKT/mTOR and Ras/Raf/MEK/EKT that drive neoplastic growth. Howbeit due to resistance formation through genetic variation, dose dependent toxicity and feedback pathway activation a need for alternate targeting pathways or receptors needs to be identified.

Prediction and Identification of eEF1A Methyltransferase:

Methylation is the most common Post Translational Modification (PTM) of proteins and is mostly introduced by site specific S-adenosyl methionine (AdoMet) dependent methyltransferase (MTase) enzymes. The methylation of lysine (Lys) and arginine (Arg) are the most studied type of protein methylation. Also, the residues such as glutamine and histidine, the N and C termini of proteins are also subjected to methylation. The human genome predicts to encode around 200 MTases. The 7βS also called the seven beta strand MTase is the largest group and encodes around 130 human enzymes. Following SET (Su(var) 3-9, Enhacer of Zeste, Trithorax), the second largest group which accounts for about 50 human proteins. The lysine methylation is catalysed by lysine methyltransferases (KMT's) by addition of one, two or three ethyl groups to the Ɛ-nitrogen side chain of lysine forming methylated derivatives. The eEF1A methylation sites have been known in yeast and mammals for decades and it was only recently found out about the methyltransferases targeting eEF1A.

The human eEF1A MT's was discovered through several different techniques such as *in vitro* methylation assays and gene knockouts. METTL10 was the first human eEF1A MT's to be discovered being responsible for Lys318 trimethylation [9]. Earlier nine novel and site specific MT's were discovered which targeted the eEF1A, five in yeast and four in humans. The enzymes involved in methylation at Lys36, Lys79, Lys165, Lys318 were already predicted and found to act on residues in all three structural domains, although predominantly in domain 1 [10]. Recently two studies showed that the METTL13 enzymes were involved in the methylation of human eEF1A- trimethylation at N-terminal and dimethylation at Lyss55. In a study carried out by Jakobsson showed that METTL13 (Methyltransferases Like Protein-13) were responsible for targeting and methylation of N terminus and Lys55 of eEF1A [11]. Using a wide range of experimental approaches, they unravelled the function of human MTase, showing that it methylates eEF1A and modulates mRNA translational in codon specific manner. METTL13, an uncharacterised member of 7βS family, was the responsible KMT for generating methylation of eEF1A at lysine 55.

Based on the following analysis by Liu *et al* [2] the identification of the enzyme was made: (a) Liquid chromatography-tandom mass spectrometry (LC-MS/MS) of methylated eEF1A purified from seven cell lines harboured dimethylation at K55. (b) eEF1AK55me1-3 state specific antibodies selectively recognised methylation on eEF1A peptides in addition to anti-eEF1AK55me2 antibody. (c) In a gene editing coupled biochemical screening using 107 known and putative KMT's along with CRISPR-cas9 tagged with 3 independent small guide RNA's (sgRna) per KMT gene. These generated 322 individual U2OS cell lines, where it was systemically probed with eEF1AK55me2 antibody to determine the gene deletion resulting in loss of eEF1A methylation signal. Strikingly out of 107 KMT's METTL13 expression was the one that reduced eEF1AK55me2 signal. Collectively from these results, it was clear that METTL13 was required for the maintenance of physiologic levels of eEF1AK55me2 in cells for the catalytic activity dependent manner and bonafies it as the KMT that methylates eEAF1A at K55 *in vitro*.

RAS pathway:

RAS proteins are proto-oncogenes belonging to a class called small GTPase and are frequently mutated in human cancers. Ras proteins are modulated by guanine nucleotide exchange factors (GEFs) leading to GTP binding through GDP dissociation. The restoration activity of GTPase is carried out by GTPase-activating proteins (GAPs) paramounting in switching off of Ras signals. Divergent Ras functions are linked with cancer and in tumors through mutation at codons 12,13 or 61 typically [12]. These favours continuous GTP binding constituting to permanent Ras activation. RAS is involved in activation of various pathways.

The PI3K pathway plays an elementary role in growth, metabolism and survival of oncogenic cells in several human cancers. The PI3K signalling pathway is triggered by G protein-coupled receptors (GPCR) along with cell surface receptors such as Epidermal Growth Factor Receptor (EGFR), Fibroblast Growth Factor Receptor (FGFR), and Insulin like Growth Factor 1 Receptor (1GF-1R) [13]. Phosphodyl inositol diphosphate (P1P2) phosphorylates to phosphatidyl inositol triphosphate (P1P3) leading to initiation of oncogene AKT through PI3K. This pathway is negatively regulated by Phosphatase and tensin homolog (PTEN). The cell proliferation and metabolic pathway effetors are phosphorylated by a seine/threonine specific protein kinase. AKT Stimulates the mammalian target of rapamycin (mTOR) by inhibition of tuberous sclerosis complex- 2 (TSC2) leading to high protein synthesis, thus making AKT a key model for linking cell growth, metabolism and apoptosis. mTOR is a serine/threonine

kinase made by two distinguishable complexes, mTORC1 and mTORC2. Both the complex needs to be inhibited for active control over cancer growth. Preferential inhibition of mTORC1 alone exerts a negative feedback loop on PI3K or even through reflex activation of AKT by mTORC2. One of the key mechanisms of dysregulation of PI3K pathway is due to insulin and IGF-1 signal or loss of PTEN or due to genetic alteration [14].

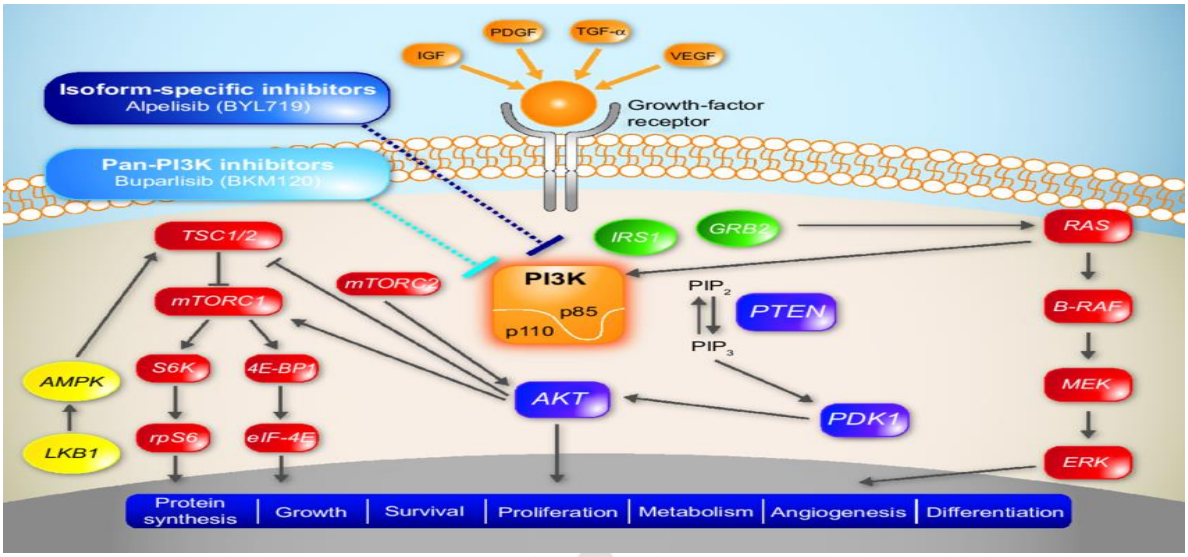

Figure No. 1: **Overview of PI3K/AKT/mTOR pathway and drug targets.**

AMPK: AMP-dependent protein kinases; GF: growth factor; GRB2: growth factor receptor-bound protein 2; IRS1: Insulin receptor substrate 1; PDK1: phosphoinositide-dependent kinase 1; PIP2: phosphatidylinositol 4,5-bisphosphate; PIP3: phosphatidylinositol 3,4,5-triphosphate. Adapted from "PI3K inhibitors as new cancer therapeutics: implications for clinical trial design" by Cristian Massacesi, OncoTargets and Therapy 2016:9 203–210.

The other well studied pathway is the Raf/MEK/ERK pathaway, also known the mitogen-activated protein kinase. These kinases are essential for normal physiological and oncogenic development. RAS oncoprotein is involved in the regulation of growth factor-induced cell survival in cancer cells [15]. It mainly constitutes of 3 family genes KRas, NRas and HRas in which KRas remains to be highly elucidated in cancer cells. Activated Ras is involved in the phosphorylation of Raf/MEK/ERK, PI3K/AKT/mTOR and Ral guanine nucleotide exchange factors (Ral-GEFs) [16,17]. Raf constitutes of 3 isoforms ARaf, BRaf, CRaf and is involved in phosphorylation of MEK. The protein MEK too exits in two isoforms MEK1 and MEK2, which are known to activate ERK. Mutation in MAP3K1 have been noted to lead to tumurogenesis [18]. Another pathway dysregulated was found to be ERK1/2, which leads to nuclear

transcription and notably changes in cell survival, proliferation, motility and angiogenesis[19-21].

The association between the PI3K and MAPK pathway is a multiplex and still unclear. A remarkable level of crosstalk have been noticed between both the pathways [22] and the inactivation of one cascade leads to the activation of other and vice-versa which promotes to downstream signalling for cellular survival.

Pancreatic Carcinoma:

Pancreatic Ductal Adenocarcinoma (PDAC) is one of the most common malignancy of pancreas. Complete surgical removal of tumour is the only chance for cure, as it is an aggressive and difficult malignancy to treat. Though the advancement of knowledge on tumour biology, progress in diagnosis and management still the prognosis remains strikingly poor. On a meta analysis of human PDAC datasets, an upregulation of METTL13 mRNA levels and eEF1AK55me2 protein levels were established [2]. Immunohistochemistry (IHC) which is the most common application of immunostaining for selectively identifying protein in a cell of tissue section by exploiting the principle of specific antibody-antigen binding [23], showed a clear pattern of high signals of METTL13 and eEF1AK55me2 in PDAC datasets of pancreatic tissues and while METTL13 protein expression was largely undetected in by IHC in normal tissues. It was evidently clear that METT13 is not expressed in normal tissue but highly in oncogenic cell, in this regard the depletion of RPE-1 cell line didn't show much impact on the proliferation of the non-transformed cell but the PDAC T3M4 cell line showed marked reduction in proliferation of cancerous pancreatic cell lines. This clearly indicates the METTL13 methylation of eEF1AK55 in PDAC cell proliferation [2,24]. In pancreas the expression of eEF1A2 gene, located on chromosome 20q13 is significantly reactivated in cancers [25], since the highly abundant eEF1A1 isoform of eEF1A is essential for mRNA translation. 83% of the pancreatic cancer displays increased expression of eEF1A2, so K55 was tested in context of eEF1A2 function of proliferation of T3M4 cells which on deletion lead to moderate reduction in overall eEF1AK55me2 levels [26]. These focuses on the fact that METTL13-dependent eEF1A K55 dimethylation increases the GTPase activity, which may boost translation elongation and thereby increase protein synthesis.

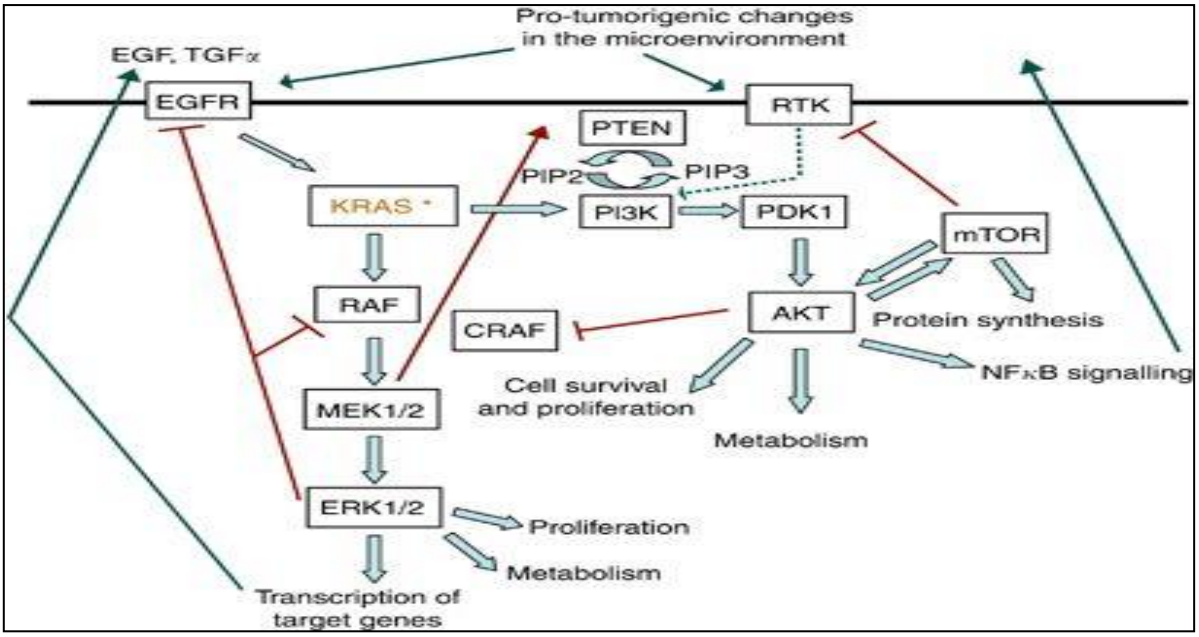

Figure No. 2: **An overview of tumuorogenic KRAS-driven PI3K/AKT/PDKI and RAF/MEK/ERK signalling networks in pancreatic cancer.**

The PI3K-PDKI-AKT pathways are engaged by mutationally activated oncogenic KRAS which drives the cancer initiation, progression and maintainance. Additionally, the canonical mitogen-activated protein kinase pathway via RAF-MEK1/2-ERK1/2 is also activated. Through positive feedback mechanism, KRAS activates the epidermal growth factor receptor (EGFR) and also with receptor tyrosine kinases (RTKs) through autocrine and paracrine stimuli. Negative feedback exists at various levels as loops inhibitory, as well as activating cross-signalling. Arrows in green depicts activating protumourigenic signalling connections; solid lines headed by a vertical line in red shows inhibitory anti-tumourigenic pathways. Activating anti-tumourigenic feedback loops are depicted in red arrows. The asterisk (KRAS*) represents the mutational activation of KRAS. Adapted from **"**Oncogenic KRAS signalling in pancreatic cancer" by S Eser, British Journal of Cancer (2014) 111, 817–822, doi: 10.1038/bjc.2014.215.

The main driving force behind the PDAC is the oncogeneic KRAS signalling [27]. The tumour entities such as PDAC, colon cancer and NSCLC differ in KRAS driven signal ling network. Conventional chemotherapy has limited effect in pancreatic cancer [28]. Novel targeted therapeutic strategies such as PI3K/AKt inhibitor and MAPK pathway inhibitors (e.g., Omipalisib, Dactolisib, and Ttrametinib) had efficiently inhibited growth in vivo on genetically engineered KRAS driven tumors and patient-derived primary PDAC xenotransplantation

models [29-30]. So, methylation of eEAF1A by METTL13 is required for KRAS driven pancreatic tumourogenesis and depletion of METTL13 results in tumour growth and it sensitizes cancer cells to PI3K/mTOR inhibitors.

Lung Carcinoma:

Lung cancer is one the most common cancer both in terms of mortality (1.76 million annual deaths) and incidence (2.09 million new cases estimated in 2018). Non-small cell lung cancer (NSCLC) comprises about 80% of all lung cancer cases. When the patients are diagnosed the majority of patients have already developed advanced disease and overall survival rates barely exceed 18 months from diagnosis. As in pancreatic cancer cell lines, a number of genetic alterations have been described in NSCLC, being Kristen Rat Sarcoma viral Oncogene (KRAS), Epidermal Growth Factor Receptor (EGFR) and Anaplastic Lymphoma Kinase (ALK) which were most commonly altered during oncogenes acting as tumour genomic drivers [31]. EGFR and ALK have shown to have high clinical efficacy towards targeted therapies [32]. In contrast, the KRAS-MAPK pathway is downstream of EGFR signalling and though do not respond to EGFR tyrosine kinase inhibitors (TKIs). This can be due genetic alterations of EGFR or Echinoderm microtubule-associated protein-like 4 (EML4) ALK translocation or because of acquired mutation after treatment and leading to drug resistance to EGFR TKIs and monoclonal antibodies [33]. Some data suggests that even when EGFR is inhibited KRAS mutation results in persistent activation of the EGFR-RAS-RAF-ERK-MEK pathway [34].

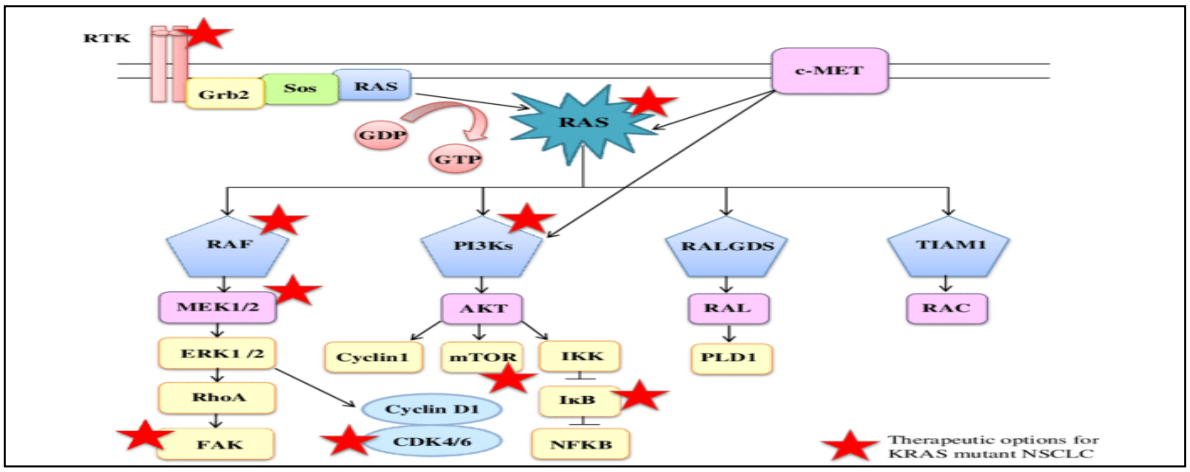

Figure No. 3: **RAS downstream signalling pathways and potential options for therapeutic intervention in lung adenocarcinoma.**

RAS-GTP (active form) and RAS-GDP (inactive from) are two alternative states of RAS proteins which regulates downstream signalling. RAS-GTP complex are involved in activation of RAF-MEK-ERK, PI3K-AKT-mTOR pathway, these signalling cascades are in turn triggered by coupling of several growth factor receptors like EGFR leading to activation of KRAS. Adapted from "KRAS oncogene in non-small cell lung cancer: clinical perspectives on the treatment of an old target" by Marta Román, Molecular Cancer (2018) 17:33 https://doi.org/10.1186/s12943-018-0789-x.

Till date, direct targeting at KRAS has not proven to be successful. Different strategies provided proof-of-concept on direct inhibition of KRAS such as compounds that target guanine nucleotide binding pocket (SML-8-73-1) [35] or irreversible allosteric inhibitor of G12C RAS [36] (most frequent mutation in KRAS mutant NSCLC) but long term efficacy and toxicity stands as hurdles. Few other studies tried to target PI3K-AKT-mTOR pathway but therapy with PI3K inhibitors were inefficient in preventing KRAS mutations as the RAF-MEK-ERK pathways can hijack tumour growth through compensatory mechanisms. Engelman et al suggested dual inhibition of PI3K/AKT/mTOR and BRAF/MEK/ERK pathways to be an effective approach to block KRAS signalling completely [37-38]. Studies using PI3K combined either with MEK or mTOR inhibitors are under evaluation.

Bladder carcinoma:

There has been an increased incidence in bladder cancer in the last few decades. Because of its poor prognosis and limited efficacious treatment options, it still remains a major clinical challenge to prevent the recurrence. In most of the carcinomas, multiple changes in the oncogenes and suppressor genes takes place for which novel markers are to be identified for early diagnosis and modelling targeted therapies. METTL13 protein was found to be one such marker, which was aberrantly expressed in various human cancers and oncogenic pathway. A Nature Scientific Reports study, have identified METTL13 as a specific tumor-suppressor protein in bladder cancer, which has a potential to be developed as a marker for prognosis, prevention and treatment. Zhang et al have been found to report low level of expression of METTL13 protein in bladder cancer cell lines and tissue samples when compared with the normal tissues and cell lines. This was in contrary to the study made by Atsushi Takahashi et al who found that most of the human cancer cell lines had overexpression of METTL13 protein and were potently involved in tumorigenesis *in vivo* [39]. But they were consistent with their findings that METTL13 was downregulated in bladder carcinoma, as they found low

expression of METTL13 as the disease progressed. To support the underlying mechanism they transfected uroepithelial and urinary bladder cell lines with wild-type METTL13 (amplification expression vector) which notably inhibited cell proliferation and METTL13 siRNA (knockdown expression vector) showed remarkable inflation in cell proliferation [8]. As we know many studies have pointed out the involvement of FAK/PI3K/AKT/mTOR signalling pathway [40] in regulation of METTL13 activity, but overexpression of METTL13 lead to obstruction of signalling pathway and conferred to anti-metastatic effects. Additionally, when overexpressed the CDK4/6 inhibitor (Palbociclib) [41], FAK inhibitor (PF-562271) [42] or AKT inhibitor (MK2206) [43] didn't contribute much in strengthening or exhausting of cell proliferation. Collectively these findings might prove to provide a new outlook on feasible ways to improve METTL13 expression level as a possible therapeutic role in management and prognosis of bladder cancer.

Hepatocellular carcinoma (HCC):

HCC is known to be the fifth most common cancer and second leading causes of cancer related death with not many treatment options. In a study by Jian Huang et al eEAF1A1 was found to influence G1 phase by promoting cyclin D1 expression through STAT1 signalling [44]. Dysregulation of AKT/mTOR and Ras/MAPK pathways was found to be involved which was first exhibited in an animal model by activated AKT and Ras proto-oncogene [45]. Tumor formation was induced through mTORC1 pathway. HCC were initially targeted with mTORC1 inhibitor Rapamycin, which showed significant delay in hepatocarcinogenesis [46]. As Rapamycin was withdrawn recurrence of tumor was found, which was most possibly due to feedback loop activation of ERK and its downstream effectors through mTORC1. A study done by Chunmei Wang and colleagues stated presence of some complex interplay between AKT/mTOR and Ras/MAPK pathway during hepatocarcinogenesis [47]. It was clear that Rapamycin partially blocked mTORC1, which lead to rise of other approaches such as use of dual mTOR/PI3K inhibitors (NVP-BEZ235, BGT226,PKI-587) [48-49] or combining Rapamycin with MEK inhibitors (AZD6244) [50]. This stimulates targeting of AKT/mTOR and Ras/MAPK provides a clear insight on effective inhibition of hepatocarcinogenesis.

Prostate cancer:

The PI3K/AKT/mTOR and androgen receptor signalling pathways are the utmost essential tumor drivers in prostate cancer [51]. This pathway is usually initiated by loss of tumor

suppressor protein PTEN. As in other cancers, AKT and mTOR kinase inhibitors have been established clinically. As discussed earlier even in prostate cancer it lead to compensatory activation of Ras/MEK/ERK pathway when treated with an AKT inhibitor (AZD7328) was initiated on a patient-derived prostate cultures. Also in a study by Dominika E. Butler, as they attempted a combination therapy with AZD7328 and two separate MEK1/2 inhibitors, further enhanced to phosphorylation of ERK1/2 [52]. These reports sufficiently suggests that single targeting of PI3K pathway is not sufficient for inhibition of prostate cancer and requires more evident combination therapy.

Breast Cancer (BC):

In women breast cancer is the second most familiar malignancy diagnosed. They are usually characterised by its hormone receptor status and expression of human epidermal growth factor receptor 2 (Her2) which are overexpressed in around 25% of BC [53-54]. Priorly endocrine therapies and monoclonal antibodies were the relative treatment available for BC. Recently PI3K pathway was detected to act as a centre for proliferation, survival and motility of BC [55]. Trastuzumab (humanised monoclonal antibody) has shown great success in inhibiting growth but it gradually developed de novo resistance [56]. In a study by Justin Cidado, an adjacent therapy with Lapatinib, a dual EGFR/Her2 kinase inhibitor [57] showed promising results. Some studies provided evidences on dual therapy with Lapatinib and Trastuzumab to be more efficient in inhibiting Her2 kinase [58].

CONCLUSION

More than 30 percent of all human cancers, including 95 percent of pancreatic cancers and 45 percent of colorectal cancers are driven by mutations of the *RAS* family of genes. NCI established the RAS initiative in 2013 to explore innovative approaches for attacking the proteins encoded by mutant forms of *RAS* genes and to ultimately create effective, new therapies for *RAS*-related cancers. Up-regulation of protein translation is a hallmark of many oncogene-driven tumors and is essential to sustain neoplastic growth; however, the mechanisms underlying this increase in protein synthesis and whether it represents a potential therapeutic vulnerability remain incompletely understood. We also explored the effect of METTL13 on cellular migration and invasion in bladder cancer cells. FAK acts as an important protein in cell-ECM interactions that affect cell proliferation, migration and metastasis. Phosphorylated FAK acts as a scaffold and primarily regulates the focal adhesion signalling

related to cell adhesion to the ECM and MMP-mediated matrix degradation. Overexpression METTL13 will inhibit bladder cancer cell migration and invasion. Many studies have indicated that the FAK/PI3K/AKT/mTOR signaling pathway is involved in the regulation of MMP-2 and MMP-9 activity. The decreased activity of the β-catenin transcription complex might contribute to the reduction in MMP2 and MMP9 gene expression. METTL13 downregulated the level of FAK phosphorylation, the level of AKT phosphorylation, β-catenin expression and MMP-9 expression as well as reduced the damage to tissue by MMP. Moreover, METTL13 overexpression could reduce the cell migration and invasion ability and whether FAK inhibitor (PF-562271) or AKT inhibitor (MK2206), did not strengthen or weaken the effect. METTL13 overexpression inhibited FAK/AKT/β-catenin signaling and contributed to the anti-metastatic effects. Whether METTL13 played a role in cell migration and invasion through directly regulate FAK/AKT/β-catenin signaling need also to be further explored. Collectively, these findings could be highly relevant in the clinical management and therapy of human cancers. METTL13 expression downregulated in cancer tissues, which might be significant to the diagnosis of bladder cancer. Moreover, the cell proliferation, migration and invasion of cancers are dependent of METTL13 inhibition. If we think of ways to improve METTL13 expression level, it may play a therapeutic role on the progress of bladder cancer. Few studies have examined METTL13 in cancers. Thus, our review might provide a new perspective on the role of METTL13 in tumorigenesis and metastasis. The possible therapeutic role of METTL13 in the management of human cancers is a crucial aspect that merits future research.

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