Differential expression of methyltransferases METTL1, METTL6 and METTL10 is associated with decreased overall survival in pancreatic ductal adenocarcinoma

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Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal cancers affecting man. We performed global differential gene expression analysis on a public dataset consisting of resected tumors from a cohort of 79 patients with PDAC (1). When comparing the transcriptomes of these tumors based on overall survival greater or less than 1 year from diagnosis, we found that methyltransferase-like 6 and methyltransferase-like 10, METTL6 and METTL10 were among the most differentially expressed genes transcriptome-wide and their expression was significantly associated with poorer survival. METTL6 is a tRNA methyltransferase for the m³c RNA modification (2), and METTL10 is a lysine methyltransferase for the eukaryotic translation factor EF1A1 (3). Analysis of a separate dataset (4) revealed that a related enzyme, METTL1 (5, 6), was also differentially expressed in patients with PDAC when stratified based on survival. These findings implicate METTL1, METTL6 and METTL10 as potential therapeutic targets in a disease with zero available targeted therapies, a median survival of 6 months and a 3-5% 5-year survival rate (7).

Keywords: pancreatic adenocarcinoma, PDAC, METTL1, METTL6, METTL10, systems oncology, discovery of targeted cancer therapeutics, differential gene expression, m³C, m⁷G.

Introduction

Pancreatic adenocarcinoma (PDAC) is the fourth leading cause of cancer death in the United States (7). In 2012, 44,000 people were diagnosed with PDAC and 37,000 of those patients died from the disease (7). PDAC is projected to be the second leading cause of cancer death in the United States by 2030 (8). Treatment includes resection if operable, and is the only potentially curative treatment (9,10). Gemcitabine is considered the single chemotherapeutic agent of choice, demonstrating a survival benefit over the previous drug of choice, fluorouracil (11-14). The addition of albumin-bound paclitaxel, nab-paclitaxel to gemcitabine demonstrated a significant improvement in median progression-free survival, from 3.7 months in the gemcitabine-alone group to 5.5 months in the gemcitabine plus nab-paclitaxel group (15). FOLFIRINOX, which includes oxaliplatin, irinotecan, leucovorin, and fluorouracil has also shown superiority to gemcitabine, with one trial demonstrating a median progression-free survival of 6.4 months in the FOLFIRINOX treated group as compared to 3.3 months median PFS in the gemcitabine group (16). Both FOLFIRINOX and the addition of nab-paclitaxel come with increases in toxicity (15,16). Pemetrexed (17), erlotinib (18), a tyrosine kinase inhibitor, capecitabine (19), bevacizumab (20), as well as platinum agents such as oxaliplatin (21) have all been studied as adjuvants to gemcitabine. The addition of erlotinib to gemcitabine demonstrated a statistically significant improvement but this was one-third of a month in median PFS (12). The addition of cisplatin (22) or oxaliplatin (21) to gemcitabine did not demonstrate an improvement in median PFS, nor did the addition of bevacizumab (14), while the addition of capecitabine showed a marginal increase in median PFS from 4.3 months as compared to 3.9 in the gemcitabine alone group (19). There is a real need for the identification of specific vulnerabilities in PDAC and subsequent development of targeted therapies.

Systems-level analyses such as transcriptome profiling do not assume any specific vulnerabilities but instead ask what the most significant changes or differences between tumor and healthy tissue, between the primary tumor and subsequent metastasis, or between those who

survive longer versus those who survive shorter are (23, 24). Species-divergent biology can present difficulty in translation of discovery from mouse to human. Thus, systems-level analysis of tumor tissue from humans with pancreatic cancer, at the genomic, epigenomic, transcriptomic, or proteomic level, analyzing or measuring DNA, epigenetic marks, RNA, or protein (23-29) present the best opportunity for the development of rational and targeted chemotherapies that minimize the toxicities that accompany the use of traditional chemotherapies.

Systems level analysis of RNA can be performed using microarray chip or RNA-sequencing to measure all or nearly all of the messages produced by a cell or tumor at any given time (23, 24, 26). Comparing microarray data from a pancreatic tumor to microarray data from healthy, reference tissue will determine in an unbiased manner what the most significant changes are between a tumor and the tissue from which it arises. Similarly, comparing microarray data from the tumors of patients who survive longer versus tumors of patients who survive for shorter periods of time can determine what the most significant biological differences are that accompany patient survival at the level of the RNA message. Here, we performed global differential gene expression profiling using two separate public datasets generated using tumors from patients with PDAC, stratifying patients based on survival (1, 2). These analyses identified three enzymes of the same family, METTL1, METTL6 and METTL10 as being the most significant transcriptional differences in patients with PDAC that survive for longer periods of time versus patients that expire in less than 12-15 months. As methyltransferases, METTL1 (2), METTL6 (3) and METTL10 (5, 6) all have catalytic sites that can be targeted.

Methods

GSE85916 (1) and GSE84219 (2) were utilized for this analysis, performed using GEO2R. Patients were stratified into two groups for each analysis; survival greater or less than 15 months for GSE84219, or survival greater or less than a year for GSE85919. GSE85916, the Brussels dataset, is a cohort of 118 patients with PDAC, and the tissue studied is the tumor itself, with RNA isolated from fresh-frozen paraffin-embedded tumors; data from 79 tumors was used for this analysis. The technology used to perform transcriptional profiling is GSE85916 was Affymetrix

U219 human microarray. GSE84219 is another published dataset obtained by transcriptional profiling of tumors from 30 patients with PDAC. The technology used for GSE84219 was an Illumina HT12 WG-DASL V4.0 R2 beadchip. For our analysis, patients from GSE84219 were stratified into two groups; survival greater or less than one year. Statistical significance of differential expression of each of the METTL1 genes was determined by GEO2R. Statistical significance of difference between mean RNA expression values between longer and shorter survivors was performed using PRISM 8.1.2 (227) by an unpaired two-way t-test with Welch's correction, while the same t-test was performed as a one-way analysis for statistical analysis of the significance of differences when comparing highest or lowest expression values between survival groups rather than RNA expression values in total.

Results

Systems analysis of the tumor transcriptome has the potential to provide critical insights into the way cancers alter their transcriptional behavior (23-26). These insights can be leveraged to identify targets for therapeutic intervention in a disease with a 3-5% 5-year survival rate (7). We performed global differential gene expression analysis on a cohort of patients with pancreatic adenocarcinoma, comparing the tumor transcriptomes of patients who survived greater than one year versus the tumor transcriptomes of patients who survived less than one year (1). We found that the methyltransferases methyltransferase-like 6 and methyltransferase-like 10, METTL6 and METTL10 were the 26th and 28th most differentially expressed genes (out of 49387 transcripts detected by this analysis) in the tumors of patients with pancreatic adenocarcinoma when stratifying patients based on survival greater or less than one year (Table 1, $p = 0.0006118$ and $p = 0.0006454$, respectively). METTL6 and METTL10 expression was significantly higher in the tumors of patients who expired within one year (Figure 1A/B and Figure 2A/B, $p = 0.00013$ and $p < 0.0001$, respectively).

We assessed whether the most extreme expression levels in this cohort correlated with survival outcomes. The lowest expression of both METTL6 and METTL10 was significantly

associated with survival over one year in this cohort of patients with PDAC (Figure 1C and Figure 2C, $p = 0.0021$ and $p < 0.0001$, respectively). Higher expression of METTL6 trended towards statistical significance when correlated with survival less than one year, but higher expression of METTL10 did not similarly appear to correlate with survival less than one year (data not shown).

Next, we interrogated an additional dataset (2), again performing global differential gene expression analysis on a cohort of patients with pancreatic adenocarcinoma to compare the tumor transcriptomes of patients who survived greater than 15 months versus the tumor transcriptomes of patients who survived less than 15 months.

We found that METTL1 was among the most differentially expressed genes in the pancreatic cancer transcriptome when comparing those who survived greater than 15 months to those who expired before 15 months, ranking 319th out of the 29374 genes that were measured as being differentially expressed in the tumors of patients with pancreatic adenocarcinoma when comparing patients that survived greater or less than 15 months, transcriptome-wide (Table 1; $p = 0.0162556$). Expression of the METTL1 transcript was significantly higher in the tumors of patients that survived longer than 15 months (Figure 3A/B; $p = 0.0039$).

We also assessed whether the most extreme expression of METTL1 was associated with survival outcomes. While the lowest expression of METTL6 and METTL10 were associated with increased survival, in this case, the highest expression of METTL1 was significantly associated with survival over 15 months in this second cohort of patients with pancreatic adenocarcinoma (Figure 3C; $p = 0.0221$). The lowest expression of METTL1 was not associated with decreased survival outcomes in this patient cohort (data not shown).

Discussion

We hypothesized that systems-level analysis of tumor transcriptomes, when stratifying patients based on survival outcomes and comparing their global gene expression profiles, would provide mechanistic insights into potential therapeutic targets. We found that the methyltransferases METTL6 and METTL10 were among the most differentially expressed genes in

the tumors of patients with pancreatic adenocarcinoma, or PDAC when comparing patients based on survival greater or less than one year, by analyzing the tumor transcriptomes of a cohort of 118 patients with PDAC. Analysis of a second dataset from a cohort of patients with PDAC revealed that the methyltransferase METTL1 was among the most differentially expressed genes in the tumors of patients with PDAC when comparing patients based on survival greater or less than 15 months. METTL6 and METTL10 were expressed, on average, at higher levels in the tumors of patients that expired within one year, while METTL1 was expressed, on average, at lower levels in the tumors of patients that expired within 15 months. While higher expression of METTL1 was significantly associated with increased survival, lower expression of METTL6 and METTL10 was significantly associated with increased survival. As enzymes, METTL6, METTL10, and METTL1 each represent attractive therapeutic targets for an aggressive disease like PDAC with no available targeted therapies.

Pancreatic cancer is the fourth leading cause of cancer death in the United States, with 37,000 deaths resulting from 42,000 diagnoses in 2012 (7). It is estimated that by 2030, pancreatic cancer will be the second leading cause of cancer death in the US, only rivaled by lung cancer (8). The gold standard for single agent chemotherapeutic treatment of PDAC, gemcitabine, provides between 3-7 months of median progression-free survival based on numerous clinical trials (11-22). Addition of adjuvant agents such as erlotinib (18) and capecitabine (19) have provided only marginal increases in median PFS, and regimens like FOLFIRINOX (16) that are considered superior to gemcitabine are lauded for the ability to extend median PFS to less than 7 months. Addition of nab-paclitaxel to gemcitabine (15) provides a similar median PFS benefit to FOLFIRINOX, but both adjuvant nab-paclitaxel and FOLFIRINOX come with additional toxicity. While erlotinib can be considered targeted with respect to the family of kinases that it targets, it does not target any specific receptor tyrosine kinase vulnerabilities in PDAC (32), and thus does not represent a targeted therapy for patients with PDAC. There are zero targeted therapeutics available for PDAC, a disease conferring a median survival of 6 months at diagnosis, and the treatment of which causes significant toxicity due to non-specificity of treatment. Systems-level

analysis of the tumor transcriptome, or the entire complement of RNA message produced by the cells of a tumor, and comparing it to non-cancerous tissue, or between those with PDAC that manifest higher and lower survival capacity, has the power to reveal fundamental of tumor transcriptional behavior for the identification of specific therapeutic vulnerabilities. Importantly, the insights gained by systems-level analysis such as the differential gene expression profiling performed here are unbiased and global.

Here, METTL6 and METTL10 were both expressed at lower levels in patients with superior outcomes, and thus inhibition of either enzyme, or both, represents an attractive therapeutic avenue for further discovery. METTL6 and METTL10 are both methyltransferases and possess catalytic sites that are targetable by small molecule inhibition, or by peptide/nucleotide “decoy” analogs of endogenous substrate. In this case, METTL1 activation rather than inhibition would be sought, since it was expressed at higher levels in patients with superior survival outcomes. METTL6 catalyzes m³C on 12% of tRNAs with a potential role in interacting with the seryl-tRNA synthetase (2), while METTL10 is a lysine methyltransferase for the translation initiation factor EF1A1 as one of its known substrates, trimethylating EF1A1 at lysine 318 (3). METTL1 is a methyltransferase that catalyzes the m⁷G mark on position 46 of tRNAs, as well as on internal guanosines in microRNAs (33-35). METTL1 is important for mouse embryonic stem cell self-renewal (36), and has a potential role in control of cell migration based on knockdown of METTL1 in A549 lung cancer cells in one study (33). We previously reported that METTL1 is the third most differentially expressed gene in a cohort of 266 patients with triple negative breast cancer (TNBC) based on global transcriptional profiling based on patient survival (37). It has recently been implicated as a biomarker in hepatocellular carcinoma (HCC) (38), and has been implicated in glioblastoma multiforme as well (39). While our previous report on TNBC and the study implicating METTL1 in HCC have reported higher expression of METTL1 in tumors and correlation with poorer disease outcomes, our analysis here indicates that, at least in PDAC, higher expression of METTL1 is associated with improved survival outcomes. METTL1 can be activated by phosphorylation by the ribosomal S6 kinase RSK, or by Protein Kinase B (PKB) (40).

By comparing transcriptome data from the primary pancreatic tumors of patients with PDAC we found using global gene expression profiling the significantly differential expression of three members of a methyltransferase family in pancreatic ductal adenocarcinoma when comparing patients based on survival outcomes (1,4); one of these enzymes (METTL1) catalyzes addition of the m⁷G mark on tRNAs (5, 6, 33, 34), while another (METTL6) catalyzes the m³C modification on tRNAs (2). It is essential to determine whether METTL6 and METTL10, and/or METTL1 are specific vulnerabilities in PDAC, a leading cause of cancer death in the United States. Specific inhibition of METTL1, along with activation or enforced expression of METTL6 and/or METTL10 represents one important avenue for therapeutic target development in PDAC.

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Figure 1: METTL6 is differentially expressed in the tumors of patients with pancreatic adenocarcinoma when comparing patients that survive greater or less than 1 year, and the lowest expression of METTL6 is significantly associated with increased survival.

Figure 1A: Expression of METTL6 RNA in tumors of patients with PDAC. Statistical significance (p-value), by an unpaired t-test with Welch's correction, shown above graph.

Figure 1B: Same as in 1A, but in XY format. Each dot represents the tumor of an individual patient.

Figure 1C: The statistical significance (p-value) of the lowest expression of METTL6 and its correlation with survival greater or less than one year, determined by an unpaired t-test with Welch's correction, shown above graph.

Figure 2: METTL10 is differentially expressed in the tumors of patients with pancreatic adenocarcinoma when comparing patients that survive greater or less than 1 year, and the lowest expression of METTL10 is significantly associated with increased survival.

Figure 2A: Expression of METTL10 RNA in tumors of patients with PDAC. Statistical significance (p-value), by an unpaired t-test with Welch's correction, shown above graph.

Figure 2B: Same as in 2A, but in XY format. Each dot represents the tumor of an individual patient.

Figure 2C: The statistical significance (p-value) of the lowest expression of METTL10 and its correlation with survival greater or less than one year, determined by an unpaired t-test with Welch's correction, shown above graph.

Figure 3: METTL1 is differentially expressed in the tumors of patients with pancreatic adenocarcinoma when comparing patients that survive greater or less than 15 months, and the highest expression of METTL1 is significantly associated with increased survival.

Figure 3A: Expression of METTL1 RNA in tumors of patients with PDAC. Statistical significance (p -value), by an unpaired t-test with Welch's correction, shown above graph.

Figure 3B: Same as in 3A, but in XY format. Each dot represents the tumor of an individual patient.

Figure 3C: The statistical significance (p -value) of the lowest expression of METTL1 and its correlation with survival greater or less than 15 months, determined by an unpaired t-test with Welch's correction, shown above graph.

Differential Gene Expression of METTL Enzymes in 2 PDAC Cohorts

Gene	Rank	p-value	B	t	ID
METTL6	26/49387	0.0006118	-1.464231	-3.5621452	NM_152396
METTL10	28/49387	0.0006454	-1.493995	-3.5459491	NM_212554
METTL1	319 / 29374	0.0162556	-3.9	2.54840701	NM_005371.4

Table 4: METTL enzymes are differentially expressed in pancreatic ductal adenocarcinoma in 2 patient cohorts when stratified and compared based on survival.

This table includes the rank of differential expression with respect to all of the transcripts detected and whose expression was measured in the respective dataset, the p-value with respect to this differential expression, and two further statistics obtained from GEO2R: the B statistic, which is the log-odds that the gene is differentially expressed, and a moderated t-statistic, calculated by comparison of the two groups (in GSE85916, $</>$ 1 year; in GSE84219, $</>$ 15 months), as well as a GenBank nucleotide accession ID for the transcript detected.