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Original Article

High Mesothelin expression in pancreatic adenocarcinoma is associated with aggressive tumor features but not prognosis

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Abstract: Mesothelin is a cell surface marker expressed on most pancreatic cancers and has been associated with aggressive biology. Despite its popularity as a drug target, clinical relevance of Mesothelin expression in pancreatic cancer is unclear. We set out to define transcriptomic signatures associated with high Mesothelin expression and identify its role in tumor biology and its clinical relevance. We analyzed pancreatic adenocarcinomas in the cancer genome atlas (TCGA), (n = 145) and the results were validated using GSE62452 cohort (n = 69). We divided the cohorts into high and low Mesothelin expression by the median. High Mesothelin was not associated with progression-free, disease-free, disease specific, nor overall survival in TCGA cohort. Despite this, high Mesothelin expression was associated with high Ki67 expression and enriched all five cell proliferation-related Hallmark gene sets, but not with previously investigated pathways: TNF-alpha, PI3K, nor angiogenesis. Mesothelin expression did not correlate with MUC16 expression. The high Mesothelin pancreatic cancers demonstrated higher homologous recombination deficiency, fraction altered, and silent and non-silent mutation rates (all $P < 0.001$) that indicate aggressive cancer biology. However, lymphocyte infiltration score, TIL regional fraction, TCR richness, infiltration of CD8 T-cells, and cytolytic activity were all significantly lower in Mesothelin high tumors (all $P < 0.015$). Finally, we found that Mesothelin expression significantly correlated with sensitivity to cytotoxic chemotherapy in pancreatic cancer cell lines. In conclusion, high Mesothelin expression is associated with enhanced proliferation, depressed immune response, and sensitivity to cytotoxic chemotherapy, which may explain there was no difference in survival in pancreatic cancer patients.

Keywords: Mesothelin, pancreatic adenocarcinoma, TCGA

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a lethal malignancy with a 5 year overall survival rate of under 10% for all comers [1]. It is the fifth leading cause of cancer-related deaths in the United States. Mainstays of treatment are systemic cytotoxic chemotherapy and surgery whenever feasible. However, the majority of patients present with metastatic disease thus a limited number of patients are eligible for surgery [1, 2]. Furthermore, in patients who do undergo resection, relapse is frequent even with addition of adjuvant systemic therapy [3]. Once metastatic disease is discovered, patients are generally considered to be incurable, and treatment is aimed at palliation. Therefore, there is a dire need to gain further insight into the tumor biology of PDAC and develop novel therapies.

Mesothelin is a cell surface glycoprotein normally expressed on pleura, pericardium, and peritoneum [4, 5]. It is not expressed in the parenchyma of any vital organs and has no known physiologic role or well-define cellular

function [6]. Its only known binding partner is MUC16 (CA125) and it does not have an intracellular signaling domain [7, 8]. It is commonly expressed on several solid tumors including gastric adenocarcinoma, ovarian carcinoma, mesothelioma and PDAC [9-12]. Due to favorable differential expression, it has become a popular target for a number of directed antineoplastic therapies including monoclonal antibodies, antibody-drug conjugates, CAR-T cells, and vaccines [13-18]. Despite lack of evidence for cellular activity, Mesothelin has been associated with invasiveness and poor outcomes in multiple malignancies. Multiple cellular pathways and mechanisms have been implicated for these observations [7, 19-21]. Despite lack of clarity regarding its cellular and physiologic function, it is clear that there is a strong correlation between Mesothelin and MUC16 expression which is associated with blood vessel invasion into the tumor and abbreviated survival in PDAC [22, 23]. Furthermore, Mesothelin has also been shown to independently predict worse outcome when strongly present on immunohistochemistry staining [24]. However, there is currently no unified theory on why Mesothelin appears to act as a poor prognosticator.

In this study we sought to identify the role Mesothelin plays in PDAC tumor biology. We set out to determine the association of the level of Mesothelin expression with overall survival and transcriptomic pathways known to effect tumor aggressiveness. Specifically, we investigated Mesothelin levels in relation to the degree of proliferation, composition of microenvironment, immune response, and sensitivity to cytotoxic chemotherapy.

Methods

Clinical and transcriptomic data of TCGA and GEO cohorts in pancreatic cancer patients

Transcriptomic and mutation data of The Cancer Genome Atlas (TCGA, n = 145) of pancreatic adenocarcinoma were obtained through the cBio Cancer Genomic Portal as we previously described [25-32]. Patient demographics are included in Supplementary Table 1. Survival data of pancreatic cancer patients were obtained from the Pan-Cancer Clinical Data Resource. Clinical and transcriptomic data of the GSE62452 (n = 69) were obtained through the Gene Expression Omnibus (GEO) repository [33]. We used Log2-transformed gene expression data for all analyses.

Scores

Homologous recombinant deficiency and mutation-related, including altered fraction, single nucleotide variant (SNV) and indel neoantigens, and silent and non-silent mutations, were calculated in the TCGA cohort by Thorsson et al [34]. Gene set scores, including angiogenesis, E2F Targets, G2M Checkpoint, and Epithelial Mesenchymal Transition (EMT), were calculated by the gene set enrichment analysis (GSEA) with hallmark gene sets of the Molecular Signatures Database (MSigDB) collection, as we previously reported [35-38]. The xCell score was defined as the infiltrating fraction of the immune and stromal cells in the tumor microenvironment, which was calculated by the xCell algorithm as previously reported [36, 39, 40].

Drug sensitivity and transcriptomic data of pancreatic cancer cell lines

Thirty-five pancreatic cancer cell lines with both comprehensive gene expression and drug response (area under the curve (AUC)) data from the cancer cell line encyclopedia (CCLE) through the Depmap portal were used to assess the correlation between Mesothelin expression and the drug response [27, 41]. The AUCs were adjusted for the range of tested drug concentrations that allowed the integration of heterogeneous drug sensitivity data from the CCLE, the Genomics of Drug sensitivity in cancer (GDSC), and the Cancer Therapeutics Response Portal (CTRP) [42].

Statistical analysis

R software (v 4.0.1, R project for Statistical Computing) and Microsoft Excel (v 16 for Windows, Redmond, WA, USA) were used to analyze data and generate figures. We divided the cohorts into high and low Mesothelin groups by the median cut-off within each cohort. We calculated *p*-values using Fischer's exact test. We also employed the Kruskal-Wallis and Mann-Whitney U tests for group comparisons. We used the log-rank for survival analyses. We used a cutoff *p*-value of 0.05 to determine significance.

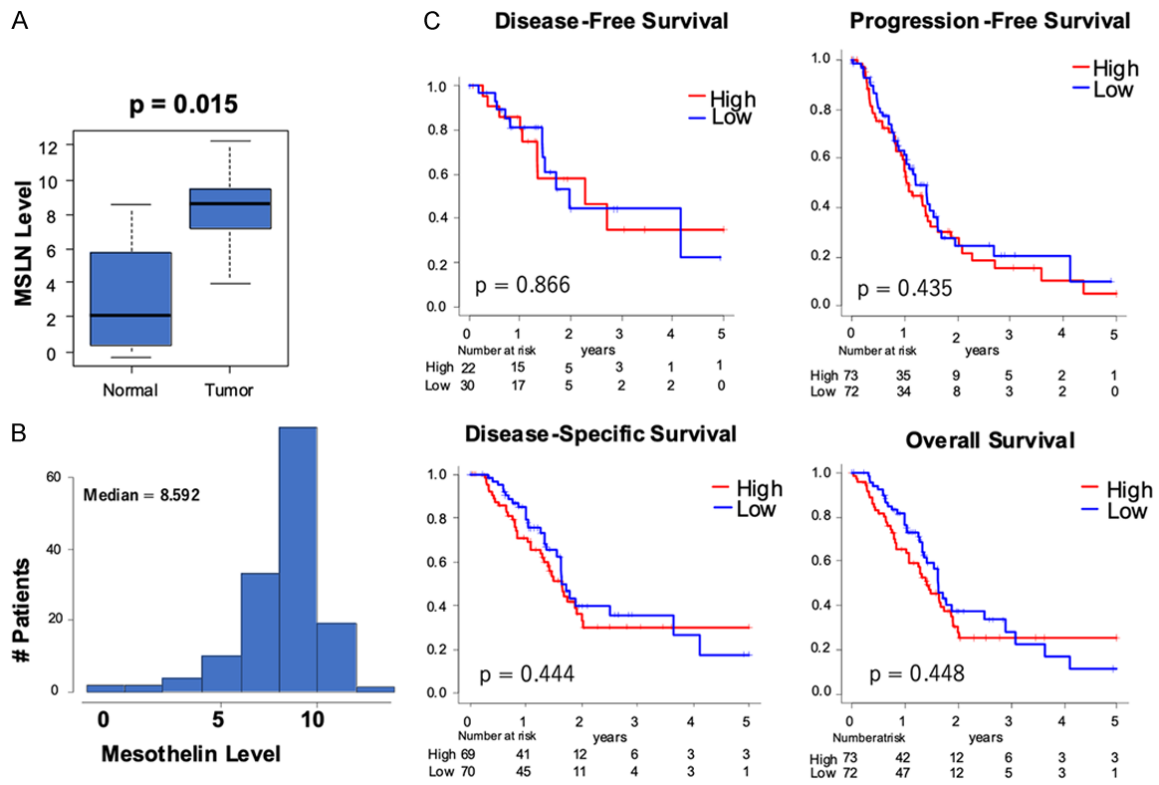

Figure 1. Mesothelin expression levels and survival. Expression levels were compared between tumor and normal pancreas tissue. As expected, Mesothelin expression was significantly higher in tumor tissue compared to normal in the TCGA cohort (A). Mesothelin expression levels in tumor tissue within the TCGA cohort form a roughly bell-shaped curve. The median expression level was 8.592 (B). The disease-free, progression-free, disease-specific, and overall survival were not different between the high and low Mesothelin groups in the TCGA cohort (C).

Results

High Mesothelin expression is not associated with abbreviated survival

It has been previously reported that Mesothelin is differentially expressed in PDAC but not in normal pancreas tissue [9]. To confirm this, we analyzed the Mesothelin expression levels between normal and tumor tissue in TCGA. Mesothelin did, in fact, demonstrate much higher levels of expression in tumor tissue compared to normal pancreatic tissue (P = 0.015, **Figure 1A**). The number of patients in TCGA cohort by Mesothelin expression was roughly bell shaped (**Figure 1B**), thus the cohort was divided into high and low groups by the median to delineate clinical relevance of Mesothelin expression. Mesothelin expression has been previously associated with worse prognosis in pancreatic cancer [22, 24]. Interestingly, in the TCGA cohort, high Mesothelin expression was not associated with abbreviated disease-free (P = 0.866), progression-free (P = 0.435), disease-specific (P = 0.444) or overall survival (P = 0.448) (**Figure 1C**). Thus, we are unable to corroborate previous reports of Mesothelin expression alone being a poor prognostic indicator.

High Mesothelin expression correlates to a highly proliferative phenotype

In order to gain more insight into the clinical relevance and tumor biology of Mesothelin expression in PDAC, we analyzed patient characteristics of high Mesothelin expressing tumors. Consistent with the lack of survival difference, high Mesothelin expression was not associated with stage or grade (P = 0.772 and P = 0.0055, respectively, **Figure 2A**). However, Ki67 gene expression was elevated in the high Mesothelin group compared to the low group (P = 0.003). Additionally, the proliferation score was elevated (P = 0.019, **Figure 2B**). To reinforce these findings, gene set enrichment anal-

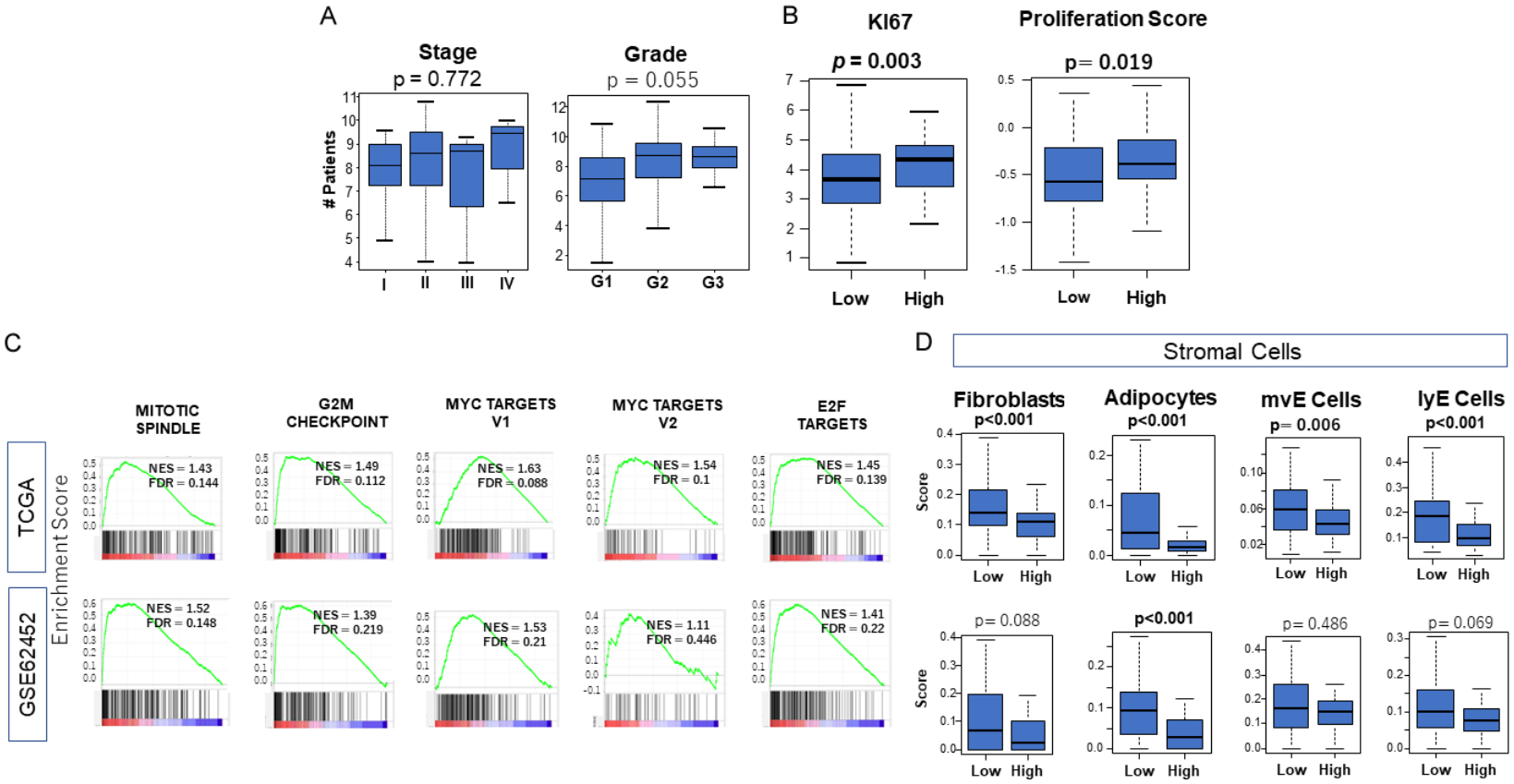

Figure 2. The number of patients with high Mesothelin were compared across stage and grade and found not to be significantly different in the TCGA cohort (A). On the contrary, Ki67 gene expression was elevated in the high Mesothelin group compared to the low group as was the proliferation score (B). Gene set enrichment analysis (GSEA) was performed in both TCGA and GSE62452 cohort and all five cell proliferation-related Hallmark gene sets were found to significantly enrich with high Mesothelin (C). Analysis of the tumor microenvironment using the xCell algorithm revealed depleted stromal cells in the high Mesothelingroup in the TCGA cohort with a similar trend in the GSE cohort (D).

ysis (GSEA) was performed in both TCGA and GSE62452 cohorts. All five cell proliferation-related Hallmark gene sets; Mitotic Spindle, G2M Checkpoint, Myc Targets v1, Myc Targets v2, and E2F Targets, were found to significantly enrich with high Mesothelin expression. All were found to have normalized enrichment score (NES) > 1.3 and false discovery rate (FDR) < 0.25 with the exception of Myc Targets v2 in GSE62452 cohort (NES = 1.11, FDR = 0.446, **Figure 2C**). Furthering the argument that high Mesothelin tumors are highly proliferative, multiple stromal cell types were found to be less present in the high Mesothelin group tumor microenvironment. Fibroblasts, adipocytes, mvE cells and lyE cells were all found to be lower in TCGA cohort (all P < 0.05). In the GSE62452 cohort, however, only adipocytes were found to have lower levels in the high Mesothelin group (P < 0.001) though there was a trend toward lower levels in the remaining cell types (**Figure 2D**).

No gene sets previously linked with Mesothelin expression are enriched in TCGA or GSE62452

Mesothelin expression and activity has been consistently linked to MUC16 and co-expression of the two has been associated with poor overall survival [23]. Thus, we sought to confirm this association in TCGA cohort. Contrary to previous reports, Mesothelin expression was not significantly correlated to MUC16 expression (r = 0.23, **Figure 3A**). Previous studies have implicated that TNF-α signaling, angiogenesis, NFkB, and PI3K-AKT signaling are associated with Mesothelin expression in pancreatic cancer [19-21]. In order to determine whether that is the case in our cohorts, we performed GSEA in both TCGA and GSE62452 cohorts. Interestingly, none of TNFα-NFkB Angiogenesis, or PI3K-AKT-MTOR pathways were enriched with high Mesothelin expression in either cohort (all NES < 1.3 and FDR > 0.25, **Figure 3B**). In addition, given that KRAS is a known driver mutation in PDAC, we questioned whether Mesothelin expression was associated with depression of KRAS signaling within the tumor which it was not (NES = -1.14 and FDR = 1, **Figure 3B**).

High Mesothelin expression is associated with suppressed anti-tumor immune response

Initially, we questioned the amount of mutational burden and neoantigen production in high versus low expressing tumors using scores pre-calculated by Thorsson et al for TCGA cohort. We found that high Mesothelin tumors had significantly elevated non-silent mutation rate, silent mutation rate, fraction altered, and homologous recombination defects (all P ≤ 0.001, **Figure 4A**). To further elucidate the clinical implications of Mesothelin, we investigated the immune landscape of tumors with high versus low expression. We employed xCell algorithms to determine tumor infiltrating lymphocyte (TIL) prevalence and overall immune response. Given these findings and given the strong differential expression of Mesothelin from normal pancreatic tissue, we hypothesized that high Mesothelin tumors would generate a strong anti-tumor immune response. However, within TCGA cohort, CD8+, CD4+, Th1, Dendritic and B-cells were all significantly lower in the high Mesothelin group (all P < 0.05). This was validated for CD8+ cells in the GSE62452 cohort (P = 0.004, **Figure 4B**). In contrast, pro-tumor immune cells such as Treg cells, Th2 cells, M2 macrophages were not different between to the two groups with the exception of Mast cells in TCGA cohort (P = 0.001, **Figure 4D**). Furthermore, lymphocyte infiltration score, T-cell receptor richness, TIL regional fraction, IFN-gamma response, and cytolytic activity score were all depressed in high Mesothelin tumors (all P < 0.05, **Figure 4C**). No indicators of anti-tumor immune response were remarkably elevated in the high Mesothelin expression group with one exception of Dendritic cells in the GSE62452 cohort (P = 0.04).

High Mesothelin expression is associated with enhanced sensitivity to cytotoxic chemotherapy

Tumors with high Mesothelin expression have aggressive features such as high proliferation, high mutational burden and depressed anti-tumor immune response. These findings appear to contradict our discovery that high Mesothelin expression alone is not associated with abbreviated overall survival. We thus investigated whether or not Mesothelin is correlated with response to chemotherapy. We queried the CCLE database for in vitro chemosensitivity assays in human-derived pancreatic cancer cell lines to four chemotherapy agents commonly used in pancreatic cancer. We found in three distinct experiments that there was enhanced sensitivity to gemcitabine (all r > 0.4)

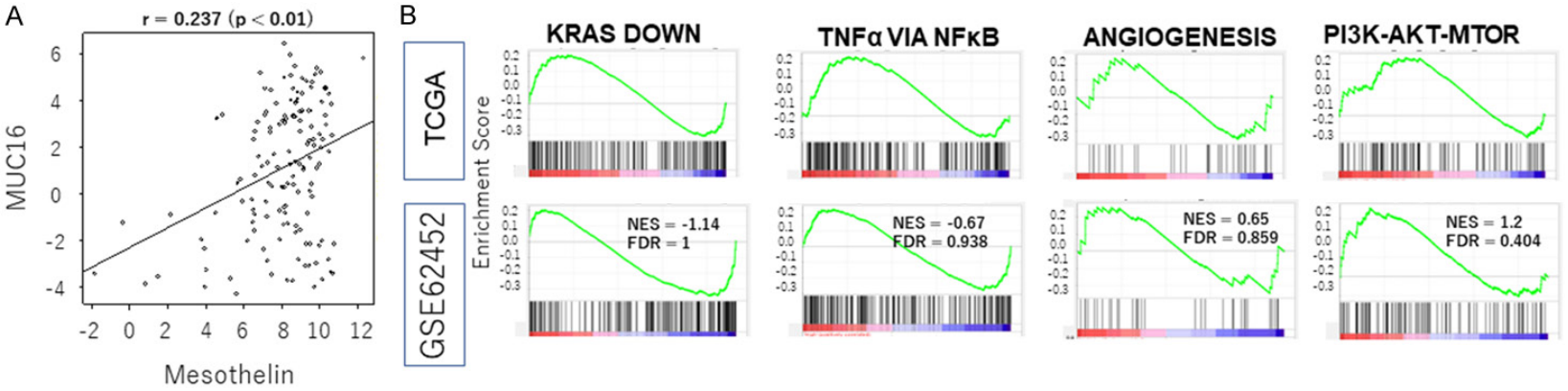

Figure 3. In the TCGA cohort, there was no correlation between MUC16 and Mesothelin expression (A). GSEA found that previously reported gene sets linked with Mesothelin function were not enriched in the TCGA or GSE cohorts (B).

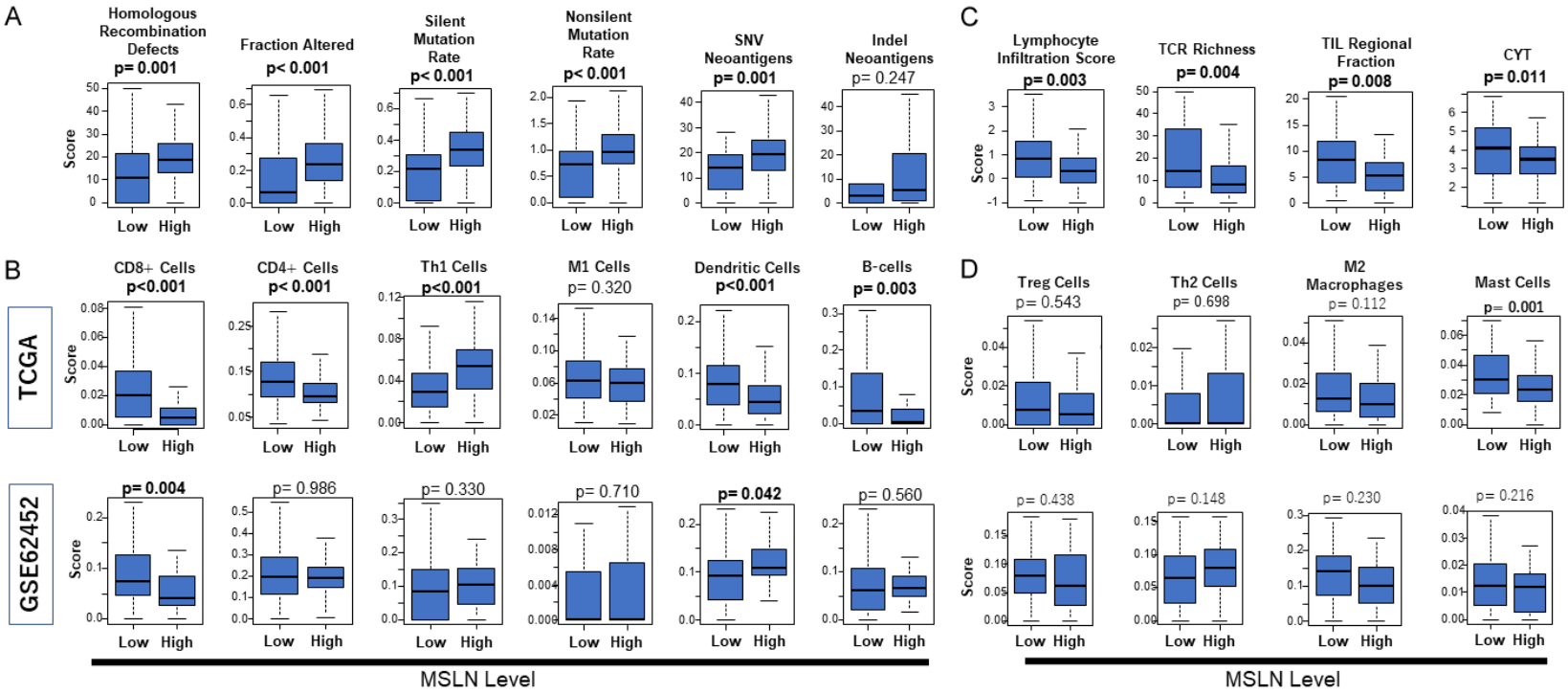

Figure 4. Thorsson score comparison in the TCGA cohort found that high Mesothelin tumors were found to have significantly elevated mutational burden and neoantigen load when compared to low Mesothelin tumors (A). When comparing high and low Mesothelin tumors on xCell algorithm analysis, high Mesothelin tumors were found to have generally lower anti-tumor immune cell infiltration in both the TCGA and GSE cohorts (B). Pre-calculated Thorsson scores revealed that high Mesothelin tumors have depressed anti-tumor immune function (C). Pro-tumor immune cell types were found not to be significantly different between high and low Mesothelin groups in both the TCGA and GSE cohorts (D).

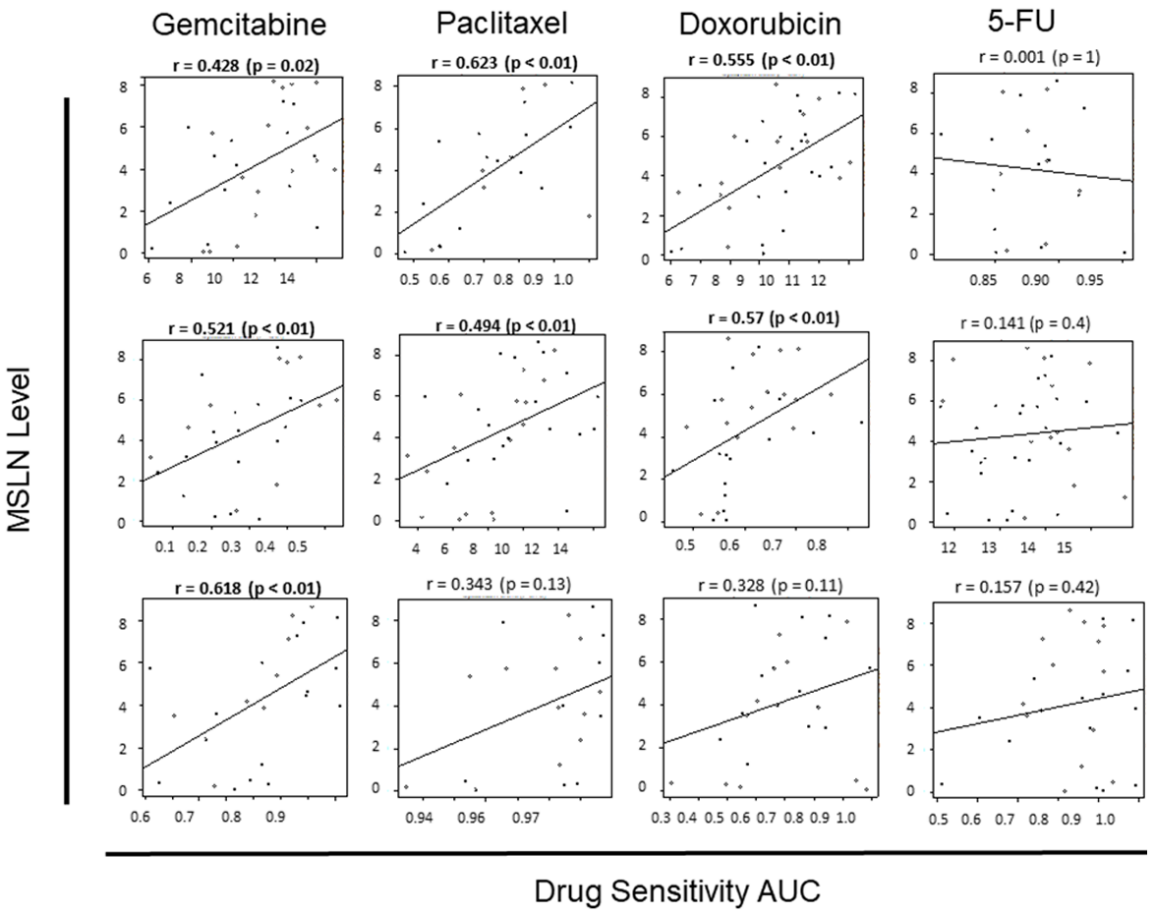

Figure 5. Analysis of in-vitro chemosensitivity assays available in the CCLE database found that human-derived pancreatic cancer cell lines demonstrated enhanced sensitivity to three chemotherapy agents commonly used in pancreatic cancer with the exception of 5-FU. The cohorts analyzed for each drug in order from top to bottom were: Gemcitabine (CTRP411863, BRDBRD-K24844714-001-24-5, GDSC21190); Paclitaxel (BRDBRD-K62008436-001-23-9, CTRP26956, GDSC21080); Doxorubicin (CTRP36599, BRDBRD-K92093830-003-30-8, GDSC1133); 5-fluorouracil (BRDBRD-K24844714-001-24-5, CCTRP25344, GDSC1179).

with higher Mesothelin expression. This was also seen for Paclitaxel and Doxorubicin, both of which were associated with enhanced sensitivity with higher Mesothelin expression in two cell lines (all $r > 0.4$). On the contrary, there was no association of Mesothelin expression with sensitivity to 5-FU across all experiments tested (all $r < 0.4$, **Figure 5**). Taken together, these data suggest that high Mesothelin expression on its own is not associated with poor prognosis due to better responsiveness to cytotoxic chemotherapy.

Discussion

Here we convincingly demonstrated that high Mesothelin expression is associated with multiple markers of high proliferation. This discovery is interesting given the lack of demonstrable intracellular signaling. Indeed, Mesothelin

expression is found in tumor types that are generally thought to be more aggressive such as PDAC, mesothelioma, gastric adenocarcinoma, and ovarian cystadenocarcinoma. These are also tumors with propensity for peritoneal and pleural spread. Binding with MUC16 likely also plays a role in this phenomenon. These facts lead one to speculate that Mesothelin expression is associated with a phenotypically distinct tumor that demonstrates aggressive characteristics. It does not appear that Mesothelin itself plays any direct role in determining tumor proliferation or aggressiveness but rather may be expressed in higher levels as tumors become more de-differentiated and fit for survival within the peritoneal microenvironment. Regardless of cause or mechanism, it is clear that tumors with high Mesothelin expression possess highly aggressive characteristics.

We also found that high Mesothelin expression is associated with a suppressed anti-tumor immune response. To our knowledge, there are no studies examining the relationship between Mesothelin expression levels and immune response. Specifically, high Mesothelin tumors had depressed CD8+ tumor infiltrating lymphocytes, lymphocyte infiltration score, TIL regional fraction, and cytolytic activity relative to low Mesothelin tumors. Furthermore, there was no evidence of any reciprocal change in the microenvironment (i.e., pro-tumor immune cells or stromal cells) that would suggest a more immunosuppressive phenotype. In light of our findings that high Mesothelin expression was also associated high proliferation, high mutational burden, and neoantigen load, one might expect the opposite result. One potential explanation for this contradiction is that the presence of high levels of Mesothelin may cause anti-tumor immune cells to recognize tumor cells as “self” and suppress their cytotoxic activity. Regardless, PDAC is well known as an “immunologically cold” tumor type [43]. Thus, in both high and low Mesothelin tumors the difference in immune response may well outweighed by other factors to make an appreciable difference on survival.

Our finding that Mesothelin expression does not have an association with prognosis in pancreatic cancer contradicts data that have been previously reported for both pancreatic and other tumor types [24, 44, 45]. The studies in question often rely on immunohistochemical (IHC) staining to define a score of Mesothelin expression. This may explain the disagreement between the fundings as IHC score may not always be consistent with quantitative gene expression level. By viewing Mesothelin expression more on a continuum, you may lose that distinction and compare tumors that are more similar in overall behavior. High Mesothelin expression is clearly associated with different tumor biology. The lack of survival difference between high and low expressors likely owes to a variety of factors such as responsiveness to chemotherapy, the heterogeneity of our study population, and the complexity of the tumor microenvironment.

In this study we gained further insight into the biologic function of Mesothelin in human tumors. In doing so, we found no pathways previously linked with Mesothelin expression and activity to be enriched in both cohorts we investigated. Given the known lack of an intracellular signaling domain, it is likely that signaling pathways previously associated with mesothelin are just those that are prominent as tumors become more aggressive. Given our findings put together with those previously reported, we theorize that high Mesothelin expression is a biproduct of further tumor mutation and de-differentiation and does not play a direct role as a driver of malignancy or tumor biology. Others have reported, however, that it does participate directly in tumor dissemination and peritoneal spread [7, 8, 20, 46]. Despite this, blockade treatment with a plain monoclonal antibody had limited effect in clinical trials [47]. Therapies to target Mesothelin have thus shifted towards immunotoxins, antibody-drug conjugates, and CAR-T cells. The lack of anti-tumor immune response seen here in high Mesothelin tumors may lend insight into the limited success of these interventions thus far. This study represents a valuable contribution to the understanding of Mesothelin’s role in pancreatic cancer and may be impactful for further development of novel therapeutics designed to target it.

This study has several limitations. This was a retrospective study done on publicly available databases and thus is subject to selection bias. Furthermore, due to the nature of the data sources, granular clinical details that

would lend more insight into the clinical relevance of our findings were lacking. Specific to the drug sensitivity analysis, each experiment was done on a different cohort, thus it is not possible to make comparisons between drug classes. On the other hand, it is noteworthy that the drugs, gemcitabine in particular, showed significant differences between cohorts. Lastly, our study was not done using any in-vitro methods rendering us unable to determine any mechanisms underpinning our findings. However, contrary to other studies of Mesothelin biology, our findings were derived from resected human tumor tissue and thus closely approximate what is happening in human PDAC tumors in-situ.

In conclusion these data demonstrate that high Mesothelin expression is associated with a highly proliferative tumor and an altered microenvironment with a depressed immune response in pancreatic cancer patients. Mesothelin expression correlated with sensitivity to cytotoxic chemotherapy drugs, which may explain there was no difference in patient survival by Mesothelin expression.

Disclosure of conflict of interest

None.

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Supplementary Table 1. Demographic and clinical data for TCGA and GSE cohorts are described

	TCGA (n = 145)	GSE62452 (n = 69)
Age (Median, IQR)	65 (35-85)	
Gender		
Male	77 (53%)	
Female	68 (47%)	
Race		
Asian	9 (6%)	
White	126 (87%)	
Black/AA	4 (3%)	
Other	4 (3%)	
AJCC pStage		
IA	3 (2%)	0
IB	9 (6%)	4 (6%)
IIA	23 (16%)	10 (14%)
IIB	103 (71%)	36 (52%)
III	3 (2%)	13 (19%)
IV	3 (2%)	6 (9%)
NA	1 (1%)	
AJCC pT stage		
T1	4 (3%)	
T2	15 (10%)	
T3	122 (84%)	
T4	3 (2%)	
NA	1 (1%)	
AJCC pN stage		
N0	37 (25%)	
N1	107 (74%)	
Nx	1 (1%)	
AJCC pM stage		
M0	68 (47%)	
M1	3 (2%)	
Unknown	74 (51%)	
Grade		
G1	21 (14%)	2 (3%)
G2	82 (57%)	35 (51%)
G3	41 (28%)	30 (44%)
G4	1 (1%)	1 (1%)
Gx		1 (1%)