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Reduced Smad4 expression and DNA topoisomerase inhibitor chemosensitivity in non-small cell lung cancer

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Abstract

Objective—Smad4 is a tumor suppressor that transduces transforming growth factor beta signaling and regulates genomic stability. We previously found that Smad4 knockdown *in vitro* inhibited DNA repair and increased sensitivity to DNA topoisomerase inhibitors. In this study, we assessed the association between reduced Smad4 expression and DNA topoisomerase inhibitor sensitivity in human non-small cell lung cancer (NSCLC) patients and evaluated the relationship between genomic alterations of Smad4 and molecular alterations in DNA repair molecules.

Materials and Methods—We retrospectively identified NSCLC patients who received etoposide or gemcitabine. Chemotherapeutic response was quantified by RECIST 1.1 criteria and Smad4 expression was assessed by immunohistochemistry. Relationships between Smad4 mutation and DNA repair molecule mutations were evaluated using publically available datasets.

Results—We identified 28 individuals who received 30 treatments with gemcitabine or etoposide containing regimens for NSCLC. Reduced Smad4 expression was seen in 13/28 patients and was not associated with significant differences in clinical or pathologic parameters. Patients with reduced Smad4 expression had a larger response to DNA topoisomerase inhibitor containing regimens than patients with high Smad4 expression (−25.7% vs. −6.8% in lesion size, $p=0.03$); this relationship was more pronounced with gemcitabine containing regimens. The overall treatment response was higher in patients with reduced Smad4 expression (8/14 vs 2/16 $p=0.02$). Analysis of data from The Cancer Genome Atlas revealed that Smad4 mutation or homozygous loss was mutually exclusive with genomic alterations in DNA repair molecules.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

Conclusions—Reduced Smad4 expression may predict responsiveness to regimens that contain DNA topoisomerase inhibitors. That Smad4 signaling alterations are mutually exclusive with alterations in DNA repair machinery is consistent with an important role of Smad4 in regulating DNA repair.

Keywords

Smad4; lung cancer; TGF β ; DNA damage; chemosensitivity

1. INTRODUCTION

Although lung cancer is the leading cause of cancer death worldwide, five-year survival remains less than 20% [1, 2]. Despite recent progress treating non-small cell lung cancer (NSCLC) with both targeted kinase inhibitors and immunotherapy [3, 4] only a minority of patients derive benefit from these approaches and most patients with metastatic NSCLC still receive conventional cytotoxic therapy at some point during their disease course [2]. Platinum based doublets form the backbone of cytotoxic NSCLC chemotherapy and produce response rates of 20–30% in unselected patients [5, 6]. While histologic subtype predicts slightly higher response to some regimens [6], there are no validated molecular markers for predicting response to specific cytotoxic therapies.

Smad4 was identified as a pancreatic cancer tumor suppressor and transduces both transforming growth factor beta (TGF β) and bone morphogenic protein (BMP) signaling [7]. Reduced Smad4 expression occurs through a combination of mutation, copy loss, and transcriptional downregulation and has been described in many malignancies including NSCLC [8–10]. In both lung and pancreatic cancer, reduced Smad4 immunostaining has been associated with reduced survival [10, 11]. The role of Smad4 as a tumor suppressor has been confirmed in animal models where Smad4 loss initiates tumor formation [9, 12], promotes the progression of oncogene-initiated lesions [13, 14], and stimulates the development of metastases [10, 15].

TGF β deletion increases genomic instability and sensitivity to ionizing radiation *in vitro* [16, 17] and Smad4 loss increases genomic instability in a head and neck cancer mouse model [12]. We previously reported that reduced Smad4 expression in NSCLC is associated with increased DNA damage, reduced DNA repair, and increased sensitivity to topoisomerase inhibitors *in vitro* [9]. In this study, we retrospectively assessed the relationship between Smad4 expression in human NSCLC and the clinical response to two chemotherapeutic drugs that have activity against DNA topoisomerase. We also used publicly available data from the cancer genome atlas (TCGA) to evaluate the relationship between genomic alterations of Smad4 and alterations in classic DNA repair molecules.

2. MATERIALS AND METHODS

2.1 Identification of a NSCLC cohort that was treated with DNA topoisomerase inhibitors

This study was approved by the University of Colorado Institutional Review Board which waived the need for informed consent. We retrospectively identified 36 patients who

received etoposide or gemcitabine (alone or in combination with other therapies) for NSCLC at the University of Colorado Hospital between 2004 to 2014. Eight patients were excluded from further analysis either because no sample was available for Smad4 immunostaining (n=6) or because there was no imaging from which a tumor response could be assessed (n=2). Two individuals received both gemcitabine and etoposide (sequentially); these treatment events were evaluated separately. For each treatment event, the use of any concurrent therapies (chemotherapy, radiation therapy) was recorded. All clinical and radiographic data were abstracted from the medical record. AJCC 7th edition staging [18] was used.

2.2. Analysis of treatment response

Lesions were evaluated on CT scan by a reviewer blinded to Smad4 status (M.Z.) using the Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 guidelines [19, 20] to categorize patients as having a complete response (CR, disappearance of all target lesions, normalization of lymph node target lesions), partial response (PR, 30% decrease in sum of longest dimension of target lesions), progressive disease (PD, 20% increase in the sum of longest dimension of target lesions or appearance of new lesions), or stable disease (SD, changes not meeting criteria for PR or PD).

2.3. Assessment of Smad4 expression

Paraffin sections were obtained from the University of Colorado Lung Cancer SPORE tissue bank then immunohistochemistry (IHC) was performed as previously described [9]. After heat-mediated antigen retrieval in 8mM sodium citrate, 0.05% Tween 20, pH 6, sections were incubated overnight at 4°C with anti-Smad4 antibody (1:100; Abcam; ab40759). Antigen was detected with biotinylated secondary antibody (1:500; Vector Laboratories; BA-1000) and VECTASTAIN avidin reagent (Vector Laboratories; PK-6100). Slides were then developed for 1 min with diaminobenzidine peroxidase (Vector Laboratories; SK-4100) and counterstained with hematoxylin. Images were acquired on a Nikon Eclipse 80i microscope with a Nikon DS-Ri1 digital camera. Smad4 expression was quantified as previously described [10] by reviewer blinded to the treatment response (K.N.). Cytoplasmic and nuclear Smad4 expression in tumor cells were assigned an intensity score (0, negative; 1, weak; 2, moderate; 3, strong) and a reactivity score (1–100%) to generate an expression score (0–300) for each staining locale. Final expression score was the average of cytoplasmic and nuclear expression scores.

2.4. Statistical analysis

Descriptive statistics were used to compare demographic and clinical data between patients with low and high Smad4 immunostaining. Linear regression was used to assess relationship between quantitative Smad4 IHC and the change of lesions in response to therapy. Fischer's exact test was used to compare overall treatment response in patients with high and low Smad4 expression. Survival was analyzed by Kaplan-Meier curves and compared with a log-rank test. Analysis was performed in Prism 5 (GraphPad, La Jolla, CA).

2.5. TCGA data and analysis

To assess the relationship between genomic alterations (mutation and homozygous deletion) of Smad4 and survival, we queried cBioportal (<http://www.cbioportal.org/>) [21, 22] using the search criteria “Smad4: mut homdel” and then applied this query to different solid tumor data sets. An identical approach was used to assess the relationship between Smad4 genomic alterations (mutation and homozygous deletion) and genomic alterations in key DNA repair molecules.

3. RESULTS

3.1 Patient and treatment characteristics

We identified 28 NSCLC patients who received etoposide or gemcitabine, had evaluable imaging, and had a sample that could be immunostained for Smad4 expression. Demographic, clinical, and pathology data are shown in Table 1. There were no significant differences based on Smad4 expression. Most patients 20/28 (71%) had advanced (stage III or IV) disease at presentation. As shown in Table 2, we identified 11 patients who received gemcitabine (6 in combination with a platinum agent, 5 as a single agent) and 17 patients who received etoposide (15 in combination with cisplatin and radiotherapy). Most patients 26/28 (93%) had advanced disease when they received these regimens.

3.2. Reduced Smad4 expression is associated with increased sensitivity to DNA topoisomerase inhibitors

We previously found that reduced Smad4 expression *in vitro* increased sensitivity to gemcitabine and etoposide [9]. Therefore we assessed the relationship between Smad4 expression and clinical response to these agents in human NSCLC patients. Treatment response was assessed by RECIST 1.1 criteria [19] and was quantified as the percent change of evaluable target lesions. Smad4 expression was assessed and quantified by IHC as previously described [9, 10]; examples of Smad4 IHC are shown in Fig. 1.

When the treatment response of patients receiving gemcitabine or etoposide containing regimens was assessed as a function of Smad4 expression, lower Smad4 expression was associated with increased treatment response (more negative percent change in lesion size, Fig. 2A). Similar to what we observed *in vitro* [9], the relationship between low Smad4 expression and chemosensitivity was more robust with gemcitabine than with etoposide (Fig. 2B–C). Interestingly, the response to gemcitabine was more pronounced in the non-first line setting where 4/7 treatment events were single agent gemcitabine (Fig. 2D). When we compared overall treatment response (CR+PR vs. SD+PD, Fig. 2E), patients with low Smad4 expression (IHC score <120) were significantly more likely to have a clinical response to gemcitabine or etoposide containing regimens than patients with high Smad4 expression (57% vs 14%, $p=0.0187$). Two patients experienced a CR and were disease free >5 years after treatment; both had low Smad4 expression and received combination therapy with etoposide/cisplatin/radiotherapy for stage IIIA disease. No relationship between Smad4 expression or chemoresponsiveness was observed in patients with molecular drivers.

Though a correlation between reduced Smad4 expression and reduced survival in lung cancer has been reported [10], we were unable to demonstrate a relationship between Smad4 expression and survival in this patient cohort (Fig. 3A). When we used publically available data (<http://www.cbioportal.org/>) [21, 22] to assess the relationship between Smad4 mutation and homozygous deletion and lung cancer survival, we found that Smad4 genomic alterations were not associated with reduced survival (Fig. 3B), although the small fraction (2.3%) of tumors with Smad4 alterations precludes a robust statistical analysis. Smad4 mutations are much more common in pancreatic cancer and low Smad4 expression by IHC has also been associated with reduced survival of pancreatic cancer patients who undergo surgical resection [11], therefore, we examined the relationship between Smad4 genomic alterations and survival in pancreatic cancer, but were unable to detect a relationship between Smad4 status and survival (Fig. 3C). In fact, no relationship between Smad4 genomic alterations and survival was detected in any of the datasets we examined including colon cancer, head and neck cancer, lung adenocarcinoma or lung squamous carcinoma (not shown).

3.3. Smad4 loss is mutually exclusive with other defects in DNA signaling molecules

Our previous data showing that reduced Smad4 expression was associated with increased DNA damage *in vivo* and reduced DNA repair *in vitro* [9], suggests that Smad4 regulates DNA repair. Therefore, we used TCGA data to assess the relationship between genomic alterations of Smad and alterations in key DNA repair molecules. Using <http://www.cbioportal.org/> [21, 22], we simultaneously examined mutations in Smad4 and selected DNA repair molecules in the pan-lung cancer dataset (Fig. 4A). While the frequency of Smad4 mutation in lung cancer is low, it is comparable with the mutation rates of key DNA repair molecules, but more interestingly, Smad4 mutations are mutually exclusive with mutations in DNA repair molecules. A similar pattern of mutual exclusivity between Smad4 mutation and mutation in DNA repair machinery was also seen when the lung adenocarcinoma and lung squamous carcinoma datasets were evaluated independently as well as in the esophageal, head and neck, and colon cancer datasets (not shown). The same analysis of the pancreatic cancer dataset suggests that Smad4 mutation may be the key driver of dysregulated DNA repair in this malignancy as very few alterations in DNA repair molecules were seen (Fig. 4B).

4. DISCUSSION

4.1. Reduced Smad4 expression is common in NSCLC

Although Smad4 mutations are rare in NSCLC, the frequency of reduced Smad4 immunostaining far exceeds that of Smad4 mutation in most aerodigestive cancers, including lung cancer, suggesting that other molecular events reduce Smad4 expression [8, 23, 24]. While we did not examine mechanisms of reduced Smad4 expression in this study, the observed frequency of reduced Smad4 immunostaining is consistent with what has been previously observed [9, 10]. We previously found that heterozygous Smad4 loss contributes to reduced Smad4 expression in NSCLC and can promote tumor growth *in vivo* [9]; while not directly assessed, our current data suggests that heterozygous Smad4 loss could contribute to DNA topoisomerase chemosensitivity. In the current study we observed

slightly lower Smad4 immunostaining in squamous carcinomas compared to non-squamous carcinomas [102 ± 21 vs 141 ± 16 (mean $\pm$ SEM), $p=0.15$]. We believe this is likely related to sample size, as in our previous publication, we did not observe any relationship between SMAD4 immunostaining and histology [9] and other investigators with have reported higher SMAD4 immunostaining in lung squamous carcinomas [10]. Similarly, because of our small study size, we could not confirm an association between reduced Smad4 immunostaining and reduced survival or pattern of disease progression [10, 11, 25, 26].

4.2. Reduced Smad4 expression may predict responsiveness to DNA topoisomerase inhibitor containing treatment regimens

We previously found that Smad4 knockdown increased sensitivity to DNA topoisomerase inhibitors *in vitro* [9]. In this study, we extended these findings to demonstrate that patients with reduced Smad4 expression have increased sensitivity to gemcitabine and etoposide containing chemotherapy regimens. Interestingly, in patients with reduced Smad4 expression, >50% demonstrated a treatment response to these regimens (Fig. 2E). This is significantly higher than the 20–40% response rate typical of first line NSCLC chemotherapy or the 10–15% response rate typical of second line chemotherapy [5, 6, 27–29]. In addition, we were unable to assess the relationship between reduced Smad4 expression and camptothecin-derived DNA topoisomerase I inhibitors as these agents are rarely used to treat NSCLC [27, 28]. One important limitation of this study is the inability to isolate the effect of reduced Smad4 expression on topoisomerase inhibitor sensitivity from any potential effects on sensitivity to platinum drugs as 26/30 evaluable treatment events included concurrent treatment with a platinum agent. However, Smad4 knockdown *in vitro* does not alter cisplatin sensitivity [9]. A similar, confounding issue exists with radiation exposure as 15/17 etoposide treatment events included concurrent radiotherapy. While there is little direct evidence regarding the role of reduced Smad4 expression and radiation sensitivity, TGF β inhibition radiosensitizes breast cancer cells *in vitro* and *in vivo* [30], thus it is possible that tumors with reduced Smad4 expression also have increased sensitivity to ionizing radiation. Finally, it is possible that our results were confounded by histology as the response rate to DNA topoisomerase containing regimens was slightly higher in squamous cell carcinoma compared to non-squamous cancers (45% vs 21%, $p=0.11$) and gemcitabine/cisplatin is likely superior to pemetrexed/cisplatin for first line treatment of squamous cell lung cancer [6]. While we cannot distinguish these possibilities in this observational study, our previous *in vitro* data support the relationship between reduced SMAD4 expression and sensitivity to gemcitabine and etoposide [9].

4.3. Smad4 may regulate DNA repair

The observation that Smad4 mutations are mutually exclusive with mutations in DNA repair machinery (Fig. 4A) is consistent with the notion that Smad4 regulates DNA repair and suggests there is little selective pressure for tumors with reduced Smad4 expression or mutation to acquire other DNA repair alterations. This is in keeping with our previous data suggesting that Smad4 loss downregulates homologous recombination repair (HRR) thus increasing dependence on non-homologous end joining (NHEJ) [9, 12]. While this contrasts with previous reports showing that Smad4 knockdown in colon or breast cancer cells decreases sensitivity to 5-fluorouracil (5-FU) [31, 32], 5-FU related lesions are repaired by

single strand DNA repair pathways [33], while damage by gemcitabine, etoposide, and camptothecin is repaired by HRR and NHEJ [34–38].

4.4. Conclusions

In sum, these data suggest that reduced Smad4 expression renders NSCLC more sensitive to treatment regimens containing DNA topoisomerase inhibitors, though it remains to be determined whether this effect can be separated from the effects of other DNA damaging agents. This study paves the way for investigating whether reduced Smad4 expression or Smad4 genomic alterations can be used as biomarkers to select treatment approaches that exploit the defective DNA repair phenotype of Smad4 deficient cells.

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HIGHLIGHTS

- Reduced Smad4 expression is common in NSCLC
- Reduced Smad4 expression is associated with sensitivity to DNA topoisomerase inhibitors
- Smad4 mutations are mutually exclusive to mutations in DNA repair molecules

Figure 1. Examples of Smad4 immunostaining in human NSCLC

Smad4 expression was scored as described in [10]. (A) Nuclear score-150, Cytoplasmic score-196, Total score-173. (B) Nuclear score-225, Cytoplasmic score-196, Total score-211. (C) Nuclear score-0, Cytoplasmic score-60, Total score-30. (D) Nuclear score-0, Cytoplasmic score-40, Total score-20. Scale bar is 100 μ m.

Figure 2. Reduced Smad4 expression is associated with increased sensitivity to topoisomerase inhibitors in human NSCLC

(A) Chemotherapeutic response of individual patients treated with gemcitabine or etoposide as a function Smad4 immunostaining. Response is shown as the percent change in the sum of target lesions longest diameter as assessed by CT scan. Smad4 IHC was quantified as described in Methods. (B–C) Patients with lower Smad4 expression are more likely to respond to etoposide and gemcitabine when drug responses are analyzed individually. (D) The response to gemcitabine was more pronounced in non-first line regimens. (E) Patients with low Smad4 expression (<120 by IHC score) are more likely to have a clinical response

to treatment with a DNA topoisomerase containing regimen. Patients with low Smad4 expression also had a greater average lesion change (-25.7% vs. -6.8% , $p=0.03$).

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Figure 3. Survival as a function of Smad4 expression and mutational status
 (A) Low Smad4 expression by IHC is not associated with reduced survival. (B–C) Smad4 genomic alterations (mutation/homozygous deletion) are not associated with reduced survival in the TCGA pan lung data set or the TCGA pancreatic cancer data set.

Figure 4. Relationship between Smad4 mutations and alterations in DNA repair molecules
TCGA data was examined by searching for “mut homdel” in Smad4 and key DNA repair molecules (PRKDC-DNA protein kinase, ATM-Ataxia Telangiectasia Mutated, ATR-Ataxia Telangiectasia And Rad3 Related, CHEK1-Checkpoint kinase-1, CHEK2-Checkpoint kinase-2, BRCA1-Breast And Ovarian Cancer Susceptibility Protein 1, BRCA2-Breast And Ovarian Cancer Susceptibility Protein 2, FANCB-Fanconi Anemia Complementation Group B, FANCE-Fanconi Anemia Complementation Group E, LIG4-DNA ligase 4, NBN-Nibrin). DNA repair genes that did not have any mutations in lung cancer are not shown. (A) Smad4

mutations are mutually exclusive to mutation in DNA repair molecules in lung cancer. A similar pattern was seen in esophageal, colon, and head and neck cancer (not shown). (B) The same analysis of the pancreatic cancer data set demonstrates a much higher frequency of Smad4 mutations and a paucity of mutations in DNA repair molecules.

Table 1

Patient demographics and clinical characteristics in NSCLC patients in this study.

	All Patients	Patients with high SMAD4 expression	Patients with low SMAD4 expression
	N = 28 (%)	N = 15 (54%)	N = 13 (46%)
Gender:			
Male	13/28 (46%)	6/15 (40%)	7/13 (54%)
Female	15/28 (54%)	9/15 (60%)	6/13 (46%)
Median age at diagnosis (years)			
	65 (48 – 85)	69 (52 – 78)	63 (48 – 78)
Median tobacco use (pack-years)			
	35 (0 – 125)	40 (12 – 70)	25 (0 – 125)
Clinical stage at diagnosis			
Stage I	5/28 (18%)	1/15 (7%)	4/13 (31%)
Stage II	3/28 (11%)	2/15 (13%)	1/13 (8%)
Stage III	12/28 (43%)	9/15 (60%)	3/13 (23%)
Stage IV	8/28 (28%)	3/15 (20%)	5/13 (38%)
Histology			
Adenocarcinoma	12/28 (43%)	8/15 (53%)	4/13 (31%)
Squamous	10/28 (36%)	3/15 (20%)	7/13 (54%)
Large cell	4/28 (14%)	3/15 (20%)	1/13 (8%)
Other	2/28 (7%)	1/15 (7%)	1/13 (8%)
Tumor Grade			
G1	1/28 (4%)	0/15 (0%)	1/13 (8%)
G2	10/28 (36%)	5/15 (33%)	5/13 (38%)
G3	13/28 (46%)	7/15 (47%)	6/13 (46%)
G4	1/28 (4%)	1/15 (7%)	0/13 (0%)
Unknown	3/28 (11%)	2/15 (13%)	1/13 (8%)
Dominant Oncogene			
<i>ALK</i>	1/28 (4%)	1/15 (7%)	0/13 (0%)
<i>EGFR</i>	5/28 (18%)	1/15 (7%)	4/13 (31%)
<i>KRAS</i>	2/28 (7%)	2/15 (13%)	0/13 (0%)
None	14/28 (50%)	9/15 (60%)	5/13 (38%)
Data unavailable	6/28 (21%)	2/15 (13%)	4/13 (31%)

Table 2

Etoposide and gemcitabine treatment events for patients in this study.

		All Patients		High SMAD4 expression		Low SMAD4 expression	
		1 st line	2 nd line	1 st line	2 nd line	1 st line	2 nd line
Etoposide							
	Etoposide + Platinum	12/28	5/28	8/15	2/15	4/13	3/13
	Concurrent XRT	10/28	5/28	6/15	2/15	4/13	3/13
Gemcitabine							
	Gemcitabine + Platinum	4/28	2/28	1/15	1/15	3/13	1/13
	Gemcitabine alone	1/28	4/28	0/15	3/15	1/13	1/13