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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 then 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.

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.

1. Introduction

Although lung cancer is the leading cause of cancer death worldwide, five-year survival remains less then 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 tranduces both transforming growth factor beta (TGFβ) 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 survivial [10,11]. The role of Smad4 as a tumor suppressor has been confirmed in animals 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].

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TGFβ 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 publically 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 2004–2014. 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 concurrent therapies 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 longest dimension of target lesions or appearance of new lesions), or stable disese (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 8 mM 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 (< 120 by IHC score) and high Smad4 immunostaining. Linear regression was used to assess relaitionship 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 1
Patient demographics and clinical characteristics in NSCLC patients in this study.

	All Patients N = 28 (%)	Patients with high SMAD4 expression N = 15 (54%)	Patients with low SMAD4 expression 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			
ALK	1/28 (4%)	1/15 (7%)	0/13 (0%)
EGFR	5/28 (18%)	1/15 (7%)	4/13 (31%)
KRAS	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%)