RESEARCH ARTICLE

Association of SMAD4 loss with drug resistance in clinical cancer patients: A systematic meta-analysis

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Abstract

Background

Drug resistance frequently led to the failure of chemotherapy for malignant cancers, hence causing cancer relapse. Thus, understanding mechanism of drug resistance in cancer is vital to improve the treatment efficacy. Here, we aim to evaluate the association between SMAD4 expression and the drug resistance in cancers by performing a meta-analysis.

Method

Relevant studies detecting SMAD4 expression in cancer patients treated with chemo-drugs up till December 2020 were systematically searched in four common scientific databases using selected keywords. The pooled hazard ratio (HR) was the ratio of hazard rate between SMAD4neg population vs SMAD4pos population. The HRs and risk ratios (RRs) with 95% confidence intervals (CIs) were used to explore the association between SMAD4 expression losses with drug resistance in cancers.

Result

After an initial screening according to the inclusion and exclusion criteria, eleven studies were included in the meta-analysis. There were a total of 2092 patients from all the included studies in this analysis. Results obtained indicated that loss of SMAD4 expression was significantly correlated with drug resistance with pooled HRs (95% CI) of 1.23 (1.01–1.45), metastasis with pooled RRs (95% CI) of 1.10 (0.97–1.25) and recurrence with pooled RRs (95% CI) of 1.32 (1.06–1.64). In the subgroup analysis, cancer type, drug type, sample size and antibody brand did not affect the significance of association between loss of SMAD4 expression and drug resistance. In addition, there was no evidence of publication bias as suggested by Begg's test.

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Conclusion

Findings from our meta-analysis demonstrated that loss of SMAD4 expression was correlated with drug resistance, metastasis and recurrence. Therefore, SMAD4 expression could be potentially used as a molecular marker for cancer resistance.

Introduction

Drug resistance in cancers contributes to the failure of chemotherapy and the subsequent cancer relapse, finally causing the death of patients [1–3]. Development of drug resistance is often associated with multiple intrinsic and extrinsic factors of cancer cells [1, 4]. Among this, signaling pathways activation plays an important role, including EGFR, Ras/MAPK, PI3K/Akt, Notch, Wnt/β-catelin and TGFβ pathways [5]. Of these, TGFβ signaling pathway regulates multiple cellular processes, including cell proliferation, differentiation, apoptosis, and specification of developmental fate during embryogenesis as well as in mature tissues [6].

The pathway ligands, such as TGFβs, BMPs and activins, bind to the TGFβ receptors to phosphorylate SMAD2 and SMAD3, forming a SMAD2/SMAD3 complex that subsequently interacts with SMAD4 to form a trimer complex, then which is translocated into the nucleus to initiate the downstream target genes transcription [7]. During cancer progression, TGFβ initially functions as a tumor suppressor, but eventually adopting promoter roles during the malignant stage [7]. It was well known that activated TGFβ induces epithelial-to-mesenchymal transition (EMT) that is often associated with cancer metastasis. However, the function of TGFβ in activating SMADs for EMT was unsure. Some studies showed that SMAD2 promotes cancer metastasis, resulting in poor survival of patients [8, 9]. Contrarily, several other studies revealed that SMAD2 suppressed EMT and cancer metastasis [10–13]. These results hence indicated that the exact role of SMAD2 in cancer is puzzling. As for SMAD3 function in cancer, most studies unanimously shown that SMAD3 promotes cancer progression and metastasis. Apart from this, a study by Michiko et al. revealed that a high frequency of SMAD4 gene mutations existed in human colorectal cancers, thus showing that inactivation of SMAD4 is important during cancer progression [14]. This finding suggested the role of SMAD4 as a suppressor gene. It is further supported by another study by Ding et al. who demonstrated an inhibition of prostate cancer growth and progression in the presence of SMAD4 [15]. Additionally, Charles reported that when SMAD4 was present in tumor cells, TGFβ had induced lethal EMT. Meanwhile, TGFβ had resulted in EMT and promoted tumor progression when SMAD4 was absent in tumor cells [16, 17]. The above studies proved that SMAD4 acts as a suppressor in the EMT process and tumorigenicity.

In the clinical studies, most of the reports showed that SMAD4 loss was correlated with cancer malignancy and prognosis. There are also several systematic reviews that studied the clinicopathological significance of SMAD4 loss in various cancers [3, 18–20], which had revealed that loss of SMAD4 was indeed associated with poorer survival and hence, is a negative prognostic indicator in patients. In agreement with this, Panagiotis et al. also found that SMAD4 inactivation had promoted the cancer progression and drug resistance of colorectal cancer in both in vitro and in vivo settings [21, 22]. However, there are other studies suggesting that SMAD4 inactivation had no significant correlation with the sensitization of pancreatic cancer cells to cisplatin or drug resistance [23, 24]. Thus, we conducted a meta-analysis of eligible studies to investigate an association between the loss of SMAD4 expression and the resistance of cancer to chemotherapy in order to clarify the exact prognostic value of SMAD4 loss in drug resistance.
Materials and methods

Publication search strategy

Systematic review of several databases was conducted in December 2020 with no lower limitation set for date of publication. The potentially relevant publications were searched in several established databases, including PubMed, EMBASE, Cochrane library and ISI Web of Science. Medical subheading (MeSH) terms related to SMAD4 (or DPC4) in combination with words related to cancer (Cancer* or Adenocarcinoma* or Carcinoma* or Tumor*), chemotherapy (or Chemoradiation or chemo* or drug), as well as terms related to patient (or patient*) were used to retrieve eligible studies.

1035 articles were extracted from the initial search using the combined MeSH terms. There were 152 articles identified from PubMed, 214 articles from ISI Web of Science, 652 articles from EMBASE and 17 articles from Cochrane library. A detailed screening process was illustrated as shown in Fig 1.

Study selection criteria

Studies were included based on the following criteria: (1) the study objects were the patients; (2) the patients had to be treated with chemotherapy or chemoradiation; (3) the studies had to detect the SMAD4 expression level in tumor tissue; (4) the results in the studies had to show the overall survival curve data or present the HRs and 95% CIs. Studies were excluded according to the following criteria: (1) studies with duplicated data or a repeated analysis; (2) letters, reviews, case reports or conference; (3) study objects were xenografted animals with patient-derived cancer cells. (4) SMAD4 mutations with unknown protein function.

Data extraction

The articles that have fulfilled both inclusion and exclusion criteria were reviewed and vital information was extracted by two investigators (X.W. and Lee S.H.) independently. Any disagreement was discussed and a consensus was reached for all issues. The following information were collected from each study: first author’s name, year of publication, cancer type, drug type, SMAD4 test, antibody brand, antibody dilution, sample size, number of SMAD4pos, number of SMAD4neg, outcome (overall survival), P value, HRs (the survival time of SMAD4neg population vs SMAD4pos population) with 95% CIs from multivariate analysis.

Quality assessment

The quality of the included studies in meta-analysis was evaluated using the Newcastle-Ottawa quality assessment scale (NOS) [25]. The scale includes eight items with three different dimensions: selections (four items, one star for each item), comparability (one item, two stars), and outcome (three items, one star for each item). Total item stars were applied to quantitatively assess study quality. A higher star meant higher quality. Inconsistencies during scoring process by the two independent researchers were discussed to reach a consensus agreement.

Statistical analysis

The pooled HRs were determined using HRs with their 95% CIs obtained from the studies. When the HR data cannot be obtained in the articles directly, a mathematical estimation based on the overall survival curve was performed according to the previously published methods demonstrated by Tierney et al. [26–28]. The pooled HR and 95% CI were used to estimate the effect of SMAD4 expression loss on the drug resistance of the cancers. A pooled HR > 1 implies that SMAD4-negative patients are resistant to the chemotherapy. The heterogeneity
**Fig 1. Methodological flow chart for the selection of papers in meta-analysis.**

**Identification**
- Records identified through database searching (n = 1035)
- Additional records identified through other sources (n = 0)

**Screening**
- Records after duplicates and errors removed (n = 715)
- Records after case/reviews and conference removed (n = 43)

**Eligibility**
- Full-text articles assessed for eligibility (n = 11)

**Included**
- Studies included in qualitative synthesis (n = 11)

- Studies included in quantitative synthesis (meta-analysis) (n = 11)

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Full-text articles excluded, with reasons (n = 32):  
No drug treatment (5 articles)  
SMAD4 mutations (7 articles)  
Surgical therapy (3 articles)  
Unrelated to SMAD4 (7 articles)  
No SMAD4 expression classification (6 articles)  
Unavailable survival data (3 articles)  
Median survival time (1 article)  
Unknown to drug treatment (1 article)

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1035 articles were identified from 4 online databases.  
992 records were excluded: 303 duplicated records and 17 error records; 111 cases/reviews and 258 conferences; 303 records unrelated to four key terms (patient*, cancer*/adeno*, chemo* and SMAD4/DPC4).  
32 records were excluded: 5 records did not include drug treatment in the SMAD4 group; 7 records involved SMAD4 mutation with unknown protein function; 3 records were under surgical therapy; 7 records were unrelated to SMAD4 expression in cancer progression and survival; 6 records were without SMAD4 expression classification in chemotherapy subgroup; 3 records were unavailable in survival data; 1 record only had median survival time without survival curve and 1 record was unknown for drug treatment.  
11 studies were finally chosen for meta-analysis. n, the number of the selected articles.

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among studies was estimated using Cochran’s Q test ($P_{\text{heter}} <0.05$, significant heterogeneity) and the $I^2$ statistic ($I^2 \leq 50\%$, no or moderate heterogeneity; $I^2 >50\%$, strong heterogeneity). The random-effects model was applied in pooling the HRs (95% CIs) to avoid significant heterogeneity ($P_{\text{heter}} <0.05$ and $I^2 >50\%$). A sensitivity analysis evaluating the stability of the results was performed by eliminating one study at a time. Publication bias was evaluated using the funnel analysis and begg’s test, with $P < 0.05$ to be considered significant. All statistical analysis was performed using the STATA software, version 16.0 (STATA Corporation, College Station, TX, USA). Meta-analysis was performed in STATA 16.0 using the `metan` package. All $P$ values were two tailed tests.

### Results

#### Study characteristics and quality assessment

The main characteristics of these studies were tabulated in Table 1. There were 2,092 patients in the included eleven studies. All studies supplied the survival time or HRs of the patients, who were divided into SMAD4$^{\text{pos}}$ and SMAD4$^{\text{neg}}$ subgroups and treated with drugs. Six out of the eleven articles studied colorectal cancer. The other articles were about pancreatic cancer as shown in Table 1. SMAD4 expression was evaluated by immunochemistry staining in ten of the studies, while only one study used the RT-qPCR to evaluate whether the SMAD4 deletion or not. Five of eleven studies used SMAD4 antibodies produced by Santa Cruz Biotechnology Inc (Dallas, TX) while three studies used SMAD4 antibody purchased from Abcam Biotechnology Inc (Cambridge, UK). The sample size of six studies is over 150. Only three studies presented information on the drug administration route and dose used. The quality of the

| Author              | Year | Cancer type | Drug type | Smad4 detection | Antibody brand | Dilution | Sample size | Smad4$^{\text{pos}}$ | Smad4$^{\text{neg}}$ | Outcome | Drug administration route | Dose               |
|---------------------|------|-------------|-----------|-----------------|----------------|----------|-------------|--------------------|-----------------|----------|---------------------------|-------------------|
| Bachet J.B.         | 2012 | Pancreatic  | gemcitabine| IHC             | Santa Cruz     | 1–50     | 249         | 87                 | 162             | OS       | N.A                       | N.A               |
| Baraniski A.        | 2011 | Colorectal  | 5-FU +oxaliplatin | IHC | Santa Cruz     | 1–100    | 190         | 125                | 65              | OS,DFS   | on days 1, 8, 15, 22 every day 36 | 2000 mg/m2/22 hours and 50mg/m2/2 hours |
| Fang Y.             | 2020 | Pancreatic  | gemcitabine| IHC             | Abcam          | 1–100    | 80          | 21                 | 59              | OS       | on days 1,8,15 for 6 times   | 1,000 mg/m2        |
| Su F.               | 2016 | Colorectal  | FULV or FOLFOX4 | IHC | Santa Cruz     | 1–150    | 174         | N.A                | N.A             | OS,DFS   | N.A                       | N.A               |
| Shin S.H.           | 2017 | Pancreatic  | 5-FU      | IHC             | Abcam          | 1–100    | 641         | 165                | 476             | OS,DFS   | N.A                       | N.A               |
| Kozak M.K.          | 2015 | Colorectal  | 5-FU      | IHC             | Santa Cruz     | 1–200    | 46          | 33                 | 13              | OS, PFS  | N.A                       | N.A               |
| Boulay J.L.         | 2002 | Colorectal  | 5-FU      | qPCR            | N.A            | 202      | 67          | 135                | OS,DFS          | infusion for 7days | 500 mg/m2           |
| Alhopuro P.         | 2005 | Colorectal  | 5-FU      | IHC             | Santa Cruz     | 1–100    | 75          | 65                 | 10              | OS, DFS  | N.A                       | N.A               |
| Ormanns S.          | 2017 | Pancreatic  | Gemcitabine +fluopyrimidine | IHC | Atlas          | 1–300    | 143         | 51                  | 92              | OS, DFS  | N.A                       | N.A               |
| Wasserman I.        | 2018 | Colorectal  | 5-FU      | IHC             | Abcam          | 1–200    | 191         | 169                | 22              | RFS      | N.A                       | N.A               |
| Herman J. M.        | 2011 | Pancreatic  | 5-FU or gemcitabine | IHC | N.A           |          | N.A         | 101                | N.A             | OS       | N.A                       | N.A               |

pos, positive; neg, negative.

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Loss of SMAD4 expression was correlated to cancer resistance

Those eleven selected articles were subjected to multivariate analysis, whereby random-effects model was used to combine the effect of the SMAD4 expression loss on chemoresistance. The pooled HR (95% CI) was calculated to be 1.23 (1.01–1.45) (Fig 2A), indicating that the loss of SMAD4 expression in the cancers resulted in chemoresistance. Since metastasis and recurrence were also correlated to cancer resistance, we also pooled the RRs (risk ratio) of metastasis and recurrence in SMAD4 subpopulation to evaluate the effect of SMAD4 expression loss on chemoresistance. The pooled RRs (95% CI) were determined to be 1.10 (0.97, 1.25) and 1.32 (1.06, 1.64) (Fig 2B and 2C). Therefore, this result is indicating a significant correlation between SMAD4 expression loss in cancers with promotion of drug resistance.

Subsequently, we analyzed the studies based on different cancer type’s subgroup. We found that the pooled HR (95% CI, \( P_{ES} \)) was 1.15 (0.88–1.41, <0.001) in pancreatic cancer subgroup while the pooled HR (95% CI, \( P_{ES} \)) was 1.46 (0.99–1.93, <0.001) in colorectal cancer subgroup (Fig 3D, Table 3). The result indicated that SMAD4 expression loss correlation with the drug resistance was independent on the cancer type.

Besides, we were also interested to know whether the correlation between SMAD4 loss and resistance is varied using different SMAD4 antibody brands. Therefore, the pooled HRs in the subgroup of SMAD4 antibody was analyzed. The results revealed the pooled HR (95% CI, \( P_{ES} \)) in SANTA CRUZ subgroup was 1.22 (0.75–1.69, 0.004), pooled HR in Abcam subgroup was 1.41 (0.85–1.97, <0.001), while HR in the other brand subgroup was 1.20 (0.79–1.62, <0.001) (Fig 3A, Table 3). The HR of each subgroup was >1 and \( P < 0.01 \), which means SMAD4 expression loss significantly promoted cancer resistance independent on the antibody brand.

To exclude the possibility that drug resistance was varied in different type of chemo-drugs, we analyzed the pooled HR in drug type subgroup. It was found that HR (95% CI) of 5-FU chemo-drug subgroup was 1.38 (0.92–1.85), HR (95% CI) of gemcitabine subgroup was 1.25 (0.61–1.89), whereas HR (95% CI) of the combined drugs was 1.36 (0.62–2.09) (Fig 3B, Table 3). All the pooled HRs of the subgroup >1 and \( P_{ES} < 0.01 \) (Table 3). This revealed that

| Author          | Year | The hazard ratio        | The risk ratio of recurrence | The risk ratio of metastasis | NOS scale (\( \ast \)) |
|-----------------|------|-------------------------|-------------------------------|-------------------------------|------------------------|
| Bachet J.B. [29]| 2012 | 0.85 0.49 1.46          | 1.06 0.81 1.38               | N.A N.A N.A                 | 8                      |
| Baraniskina A. [30]| 2011| 1.88 1.15 3.1           | N.A N.A N.A                 | N.A N.A N.A                 | 8                      |
| Fang Y. [31]    | 2020 | 2.39 1.55 3.69          | N.A N.A N.A                 | 1.19 0.58 2.45               | 8                      |
| Su F. [32]      | 2016 | 1.14 0.84 1.54          | N.A N.A N.A                 | 1.158 0.89 1.5               | 8                      |
| Shin S.H. [33]  | 2017 | 1.21 0.99 1.48          | 1.42 1.12 1.8                | N.A N.A N.A                 | 8                      |
| Kozak M.K. [34] | 2015 | 4.85 1.96 12.03         | N.A N.A N.A                 | N.A N.A N.A                 | 8                      |
| Boulay J.L. [35]| 2002 | 2.09 1.29 3.39          | 2.08 0.83 5.2                | N.A N.A N.A                 | 8                      |
| Alhopuro P. [22]| 2005 | 3.4 1.2 9.62            | N.A N.A N.A                 | N.A N.A N.A                 | 8                      |
| Ormanns S. [36] | 2017 | 1.088 0.751 1.576       | N.A N.A N.A                 | N.A N.A N.A                 | 8                      |
| Wasserman L. [24]| 2018| 1.14 0.62 2.1           | 1.54 0.97 2.45               | 1.215 0.92 1.61              | 7                      |
| Herman J. M. [37] | 2011| 1.05 0.69 1.598         | N.A N.A N.A                 | N.A N.A N.A                 | 6                      |

HR, Hazard Ratio; CI, confidence interval; RR, Risk Ratio.

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The loss of SMAD4 expression was correlated to cancer resistance. The included studies was assessed according to the NOS scale. Among 11 studies, nine scored 8, one scored 7 and one scored 6 as shown in Table 2. The result showed that all the included studies were of high quality.
# Fig 2. Forest plots of studies evaluating hazard ratios (HRs) and risk ratios (RR) of SMAD4 loss for resistance

## (A) HRs between SMAD4\textsuperscript{neg} and SMAD4\textsuperscript{pos} population

| Study ID             | HR (95% CI)       | % weight |
|----------------------|-------------------|----------|
| Bachet J.B. (2012)   | 0.85 (0.49, 1.46) | 12.39    |
| Baraniskin A. (2011) | 1.88 (1.15, 3.10) | 4.34     |
| Fang Y. (2020)       | 2.39 (1.55, 3.69) | 3.69     |
| Su F. (2016)         | 1.14 (0.84, 1.54) | 17.49    |
| Shin S.H. (2017)     | 1.21 (0.99, 1.48) | 22.68    |
| Kozak M.K. (2015)    | 4.85 (1.96, 12.03)| 0.19     |
| Boulay J.L. (2002)   | 2.09 (1.29, 3.39) | 3.81     |
| Alhopuro P. (2005)   | 3.40 (1.20, 9.62) | 0.27     |
| Ormanns S. (2017)    | 1.09 (0.75, 1.58) | 14.90    |
| Wasserman I. (2018)  | 1.14 (0.62, 2.10) | 6.85     |
| Herman J. M. (2011)  | 1.05 (0.69, 1.60) | 13.40    |
| overall (I-squared = 34.5%, p=0.122) | 1.23 (1.01, 1.45) | 100.00 |

NOTE: weights are from random effects analysis

## (B) RRs of metastasis between SMAD4\textsuperscript{neg} and SMAD4\textsuperscript{pos} population

| Study ID             | RR (95% CI)       | % weight |
|----------------------|-------------------|----------|
| Fang Y. (2020)       | 0.89 (0.42, 1.93) | 2.82     |
| Su F. (2016)         | 1.13 (0.88, 1.44) | 27.4     |
| Shin S.H. (2017)     | 1.00 (0.82, 1.24) | 38.81    |
| Kozak M.K. (2015)    | 1.23 (0.78, 1.95) | 7.91     |
| Alhopuro P. (2005)   | 0.97 (0.49, 1.91) | 3.62     |
| Wasserman I. (2018)  | 1.30 (0.97, 1.74) | 19.46    |
| overall (I-squared = 0.0%, p=0.751) | 1.10 (0.97, 1.25) | 100     |

NOTE: weights are from random effects analysis

## (C) RRs of recurrence between SMAD4\textsuperscript{neg} and SMAD4\textsuperscript{pos} population

| Study ID             | RR (95% CI)       | % weight |
|----------------------|-------------------|----------|
| Bachet J.B. (2012)   | 1.06 (0.81, 1.38) | 36.26    |
| Shin S.H. (2017)     | 1.42 (1.12, 1.80) | 41.28    |
| Pia Alhopuro (2005)  | 2.08 (0.83, 5.20) | 5.16     |
| Wasserman I. (2018)  | 1.54 (0.97, 2.45) | 17.30    |
| overall (I-squared = 30.1%, p=0.232) | 1.32 (1.06, 1.64) | 100.00 |

NOTE: weights are from random effects analysis

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### A

| Study ID | HR (95% CI) | % Weights |
|----------|-------------|-----------|
| Satola Cruz | 0.85 (0.49, 1.46) | 12.39 |
| Baranaskin A. (2011) | 1.88 (1.15, 3.10) | 4.34 |
| Su F. (2016) | 1.14 (0.84, 1.54) | 17.49 |
| Kozak M.K. (2015) | 4.85 (1.96, 12.03) | 0.19 |
| Allopuro P. (2005) | 3.40 (1.20, 9.62) | 0.27 |
| subtotal (F= 40.9%, p=0.149) | 1.22 (0.75, 1.69) | 34.68 |
| Abcam | 2.30 (1.55, 3.69) | 3.69 |
| Fang Y. (2020) | 1.21 (0.99, 1.48) | 22.08 |
| Shih S.H. (2017) | 1.14 (0.62, 2.10) | 6.95 |
| Wasserman I (2018) | 1.41 (0.85, 1.97) | 33.22 |
| subtotal (F=56.0%, p=0.103) | 1.20 (0.79, 1.62) | 32.11 |
| Other | 2.09 (1.29, 3.39) | 3.81 |
| Boulaj J.L. (2002) | 1.09 (0.75, 1.58) | 14.90 |
| Ormanns S. (2017) | 1.05 (0.69, 1.60) | 13.40 |
| Herrman J.M. (2011) | 1.20 (0.79, 1.62) | 32.11 |
| subtotal (F=40.3%, p=0.187) | 1.23 (1.01, 1.45) | 100.00 |

**NOTE:** weights are from random effects analysis

### B

| Study ID | HR (95% CI) | % Weights |
|----------|-------------|-----------|
| Triclitibine | \[\text{---}\] | 12.39 |
| Bachet J.B. (2012) | 0.85 (0.49, 1.46) | 15.25 |
| Fang Y. (2020) | 2.39 (1.55, 3.69) | 5.18 |
| Herrman J.M. (2011) | 1.05 (0.69, 1.60) | 16.26 |
| subtotal (F=69.9%, p=0.036) | 1.25 (0.61, 1.89) | 36.70 |
| Combination | \[\text{---}\] | 12.39 |
| Baranaskin A. (2011) | 1.88 (1.15, 3.10) | 6.04 |
| Ormanns S. (2017) | 1.09 (0.75, 1.58) | 17.71 |
| subtotal (F=53.3%, p=0.143) | 1.36 (0.62, 2.09) | 23.75 |
| S-FU | \[\text{---}\] | 12.39 |
| Shin S.H. (2017) | 1.21 (0.99, 1.48) | 24.37 |
| Kozak M.K. (2015) | 4.85 (1.96, 12.03) | 0.28 |
| Boulaj J.L. (2002) | 2.09 (1.29, 3.39) | 5.35 |
| Allopuro P. (2005) | 3.40 (1.20, 9.62) | 0.40 |
| Wasserman I (2018) | 1.14 (0.62, 2.10) | 9.15 |
| Su F. (2016) | 1.14 (0.84, 1.54) | 17.49 |
| Herrman J.M. (2011) | 1.20 (0.79, 1.62) | 32.11 |
| subtotal (F=28.7%, p=0.231) | 1.38 (0.92, 1.85) | 39.55 |
| Overall (F=34.5%, p=0.122) | 1.27 (1.00, 1.54) | 100.00 |

**NOTE:** weights are from random effects analysis

### C

| Study ID | HR (95% CI) | % Weights |
|----------|-------------|-----------|
| <750 | \[\text{---}\] | 12.39 |
| Bachet J.B. (2012) | 0.85 (0.49, 1.46) | 12.39 |
| Baranaskin A. (2011) | 1.88 (1.15, 3.10) | 4.34 |
| Shin S.H. (2017) | 1.21 (0.99, 1.48) | 22.68 |
| Boulaj J.L. (2002) | 2.09 (1.29, 3.39) | 3.81 |
| Wasserman I (2018) | 1.14 (0.62, 2.10) | 6.95 |
| Su F. (2016) | 1.14 (0.84, 1.54) | 17.49 |
| Herrman J.M. (2011) | 1.20 (0.79, 1.62) | 32.11 |
| subtotal (I-squared = 26.0%, P=0.24) | 1.20 (0.97, 1.43) | 67.56 |
| <150 | \[\text{---}\] | 12.39 |
| Fang Y. (2020) | 2.39 (1.55, 3.69) | 3.69 |
| Kozak M.K. (2015) | 4.85 (1.96, 12.03) | 0.19 |
| Allopuro P. (2005) | 3.40 (1.20, 9.62) | 0.27 |
| Herrman J.M. (2011) | 1.09 (0.75, 1.58) | 14.90 |
| Ormanns S. (2017) | 1.05 (0.69, 1.60) | 13.40 |
| Herrman J.M. (2011) | 1.41 (0.82, 1.99) | 32.44 |
| Overall (I-squared = 34.5%, p=0.122) | 1.23 (1.01, 1.45) | 100.00 |

**NOTE:** weights are from random effects analysis

### D

| Study ID | HR (95% CI) | % Weights |
|----------|-------------|-----------|
| Pancreatic Cancer | \[\text{---}\] | 12.39 |
| Bachet J.B. (2012) | 0.85 (0.49, 1.46) | 12.39 |
| Fang Y. (2020) | 2.39 (1.55, 3.69) | 3.69 |
| Shih S.H. (2017) | 1.21 (0.99, 1.48) | 22.68 |
| Ormanns S. (2017) | 1.09 (0.75, 1.58) | 14.90 |
| Herrman J.M. (2011) | 1.05 (0.69, 1.60) | 13.40 |
| subtotal (F= 43.9.0%, p=0.129) | 1.15 (0.88, 1.41) | 67.05 |
| Colorectal Cancer | \[\text{---}\] | 12.39 |
| Baranaskin A. (2011) | 1.88 (1.15, 3.10) | 4.34 |
| Kozak M.K. (2015) | 4.85 (1.96, 12.03) | 0.19 |
| Boulaj J.L. (2002) | 2.09 (1.29, 3.39) | 3.81 |
| Allopuro P. (2005) | 3.40 (1.20, 9.62) | 0.27 |
| Herrman J.M. (2011) | 1.14 (0.62, 2.10) | 6.85 |
| Ormanns S. (2017) | 1.14 (0.84, 1.54) | 17.49 |
| Herrman J.M. (2011) | 1.46 (0.99, 1.93) | 32.95 |
| subtotal (F= 32.3%, p=0.194) | 1.25 (1.01, 1.45) | 100.00 |

**NOTE:** weights are from random effects analysis
the cancer resistance induced by SMAD4 loss was not dependent on the type of chemo-drugs. Finally, we analyzed the sample size subgroup and found that the pooled HR (95% CI, $P_{ES}$) was 1.20 (0.97–1.43, <0.01) when sample size greater than 150, the HRs was 1.41 (0.82–1.99, <0.01) when sample size less than 150 (Fig 3C, Table 3), which means SMAD4 expression loss correlation with resistance did not varied at different sample size in studies.

**Heterogeneity**

The heterogeneity of pooled HRs in these 11 studies was tested and $I^2$ value obtained was 34.5%, $P_{heter} = 0.122$, which revealed there was no heterogeneity among these eleven studies (Fig 2, Table 2). In the drug type subgroup analysis, the $I^2$ value of 5-FU chemo-drug subgroup was 28.7%, and the $P_{heter} > 0.05$. However, the $I^2$ value of gemcitabine subgroup was 69.9% with $P_{heter} < 0.05$, indicating that there was heterogeneity among these three studies of gemcitabine subgroups (Fig 3B, Table 3). For all others subgroups, the heterogeneity of Abcam subgroup, combination subgroup and >150 subgroup was greater than 50%, however, the $P_{heter}$ was greater than 0.05 (Table 3). The heterogeneity of remaining subgroups was less than 50% and $P_{heter} > 0.05$. The result indicated that there was no heterogeneity among the studies in these subgroups.

**Sensitivity analysis**

To evaluate whether any single study had an influence on the pooled results, leave-one-out method was employed for sensitivity analysis. We sequentially removed each study and calculated the pooled HRs to evaluate the effect of an individual study on the pooled results. As shown in Fig 4, pooled HRs was stable after each study was removed sequentially, which suggested that any of these studies did not affect the pooled results significantly.

Table 3. Stratified analysis of pooled HRs for cancer patients in different subgroups.

| Variable         | No. of studies | No. of Patients | HR (95% CI)      | Heterogeneity | Model |
|------------------|----------------|-----------------|------------------|---------------|-------|
| Antibody type    |                |                 |                  | $\chi^2$      | $I^2$ | $P$ Value |
| Santa Cruz       | 5              | 734             | 1.22 (0.75, 1.69)| 0.100         | 40.900% | 0.149 random |
| Abcam            | 3              | 912             | 1.41 (0.85, 1.97)| 0.138         | 56.000% | 0.103 random |
| others           | 3              | 446             | 1.2 (0.79, 1.62) | 0.054         | 40.300% | 0.187 random |
| Drug type        |                |                 |                  | $\chi^2$      | $I^2$ | $P$ Value |
| Gemcitabine      | 3              | 430             | 1.25 (0.61, 1.89)| 0.211         | 69.900% | 0.036 random |
| 5-FU             | 5              | 1155            | 1.38 (0.92, 1.85)| 0.083         | 28.700% | 0.231 random |
| Combination      | 2              | 333             | 1.36 (0.62, 2.09)| 0.168         | 53.500% | 0.143 random |
| Sample size      |                |                 |                  | $\chi^2$      | $I^2$ | $P$ Value |
| >150             | 6              | 1647            | 1.20 (0.97, 1.43)| 0.0211        | 26.000% | 0.24 random |
| <150             | 5              | 445             | 1.41 (0.82, 1.99)| 0.177         | 53.000% | 0.074 random |
| Cancer type      |                |                 |                  | $\chi^2$      | $I^2$ | $P$ Value |
| Pancreatic cancer| 5              | 1214            | 1.15 (0.88, 1.41)| 0.038         | 43.900% | 0.129 random |
| Colorectal cancer| 6              | 878             | 1.46 (0.99, 1.93)| 0.102         | 32.300% | 0.194 random |

No., number; HR, hazard ratio; CI, confidence interval.
Publication bias

Begg’s funnel plot was used to evaluate publication bias, where no asymmetry was found in the plot (Fig 5). Meanwhile, the Begg’s test revealed the $P$ value was 0.073 (Table 4), which indicated that there was no evidence for a significant publication bias in the meta-analysis.

Discussion

SMAD4 is the downstream of TGFβ signaling and mutations associated with this gene has been reported in colorectal cancer [14, 38], head and neck carcinoma [39], seminoma germ cell tumors [40] and pancreatic cancers [41, 42]. The SMAD4 mutation resulted in gene inactivation that was correlated to tumorigenesis, metastasis, poor prognosis and radio-resistance. Although there were reports that analyzed the correlation of the SMAD4 expression loss with poorer cancer prognosis, it was still unclear whether SMAD4 expression loss was significantly related to drug resistance or not. This is the first study that used meta-analysis approach to prove that SMAD4 expression loss indeed promoted the cancers drug-resistance significantly.

So far, there are many reports showing that SMAD4 knock down had rendered the cancer cells resistant to the chemo-drugs in a xenograft model. Because the xenografts and patient data have vastly different biology and outcomes. Moreover, we cannot conclude that loss of SMAD4 expression could be potentially used as a molecular marker for cancer resistance in
clinical therapy when we use animal xenograft models. Therefore, we excluded the animal xenograft models and selected patients with SMAD4 loss under chemo-drug treatment as our study focus. In our meta-analysis included 11 studies involving 2,092 patients, the sample size was enough to conduct the analysis for the association between SMAD4 expression loss and drug resistance. It is interesting to reveal that loss of SMAD4 expression was strongly associated with a worse prognosis for OS or RFS in the patients treated with chemo-drug. The result indicated SMAD4 expression loss resulted in the cancers drug-resistance significantly.

Although our findings proved that SMAD4 loss rendered the cancer cells resistance to chemotherapy, however, there are still some limitations in this study. This is because there may still exist certain degree of bias in this study since it is not possible to completely eliminate all potential bias. Firstly, the number of studies included in the meta-analysis was not enough. Besides, some of the HRs data were extracted using the strategies reported by Tierney et al. [28], hence the data calculated from the Kaplan-Meier curve may not be as precise as

Table 4. Begg’s test for funnel plot.

| adj. Kendall’s Score (P-Q) | 23 |
|----------------------------|----|
| Std. Dev. of Score         | 12.5 |
| Number of Studies          | 11 |
| $z$                        | 1.79 |
| $Pr > |z|$                    | 0.073 |
| $z$                        | 1.71 |
| ($continuity
corrected$)   |    |
| $Pr > |z|$                    | 0.087 |
| ($continuity
corrected$)   |    |

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compared to obtaining data directly from the original article. Moreover, the SMAD4 expression level was determined only by immunochemistry and RT-qPCR. On top of that, the final antibody concentration from different brand may also be different. Therefore, the cutoff value of the result may be varied that could cause the bias. Based on these reasons, a random-effects model was adopted and subgroup analysis was performed to minimize the effect of this limitation factors.

**Supporting information**

S1 Checklist. PRISMA 2009 checklist for the manuscript. (PDF)

S1 Table. The key information of the articles related to SMAD4 mutation study. OS, overall survival; HR, Hazard Ratio. (PDF)

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