TAZ Expression as a Prognostic Indicator in Colorectal Cancer

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Abstract

The Hippo pathway restricts the activity of transcriptional coactivators TAZ (WWTR1) and YAP. TAZ and YAP are reported to be overexpressed in various cancers, however, their prognostic significance in colorectal cancers remains unstudied. The expression levels of TAZ and YAP, and their downstream transcriptional targets, AXL and CTGF, were extracted from two independent colon cancer patient datasets available in the Gene Expression Omnibus database, totaling 522 patients. We found that mRNA expressions of both TAZ and YAP were positively correlated with those of AXL and CTGF (p<0.05). High level mRNA expression of TAZ, AXL or CTGF significantly correlated with shorter survival. Importantly, patients co-overexpressing all 3 genes had a significantly shorter survival time, and combinatorial expression of these 3 genes was an independent predictor for survival. The downstream target genes for TAZ-AXL-CTGF overexpression were identified by Java application MyStats. Interestingly, genes that are associated with colon cancer progression (ANTXR1, EFEMP2, SULF1, TAGLN, VCAN, ZEB1 and ZEB2) were upregulated in patients co-overexpressing TAZ-AXL-CTGF. This TAZ-AXL-CTGF gene expression signature (GES) was then applied to Connectivity Map to identify small molecules that could potentially be utilized to reverse this GES. Of the top 20 small molecules identified by connectivity map, amiloride (a potassium sparing diuretic,) and tretinoin (all-trans retinoic acid) have shown therapeutic promise in inhibition of colon cancer cell growth. Using MyStats, we found that low level expression of either ANO1 or SQUE were associated with a better prognosis in patients co-overexpressing TAZ-AXL-CTGF, and that ANO1 was an independent predictor of survival together with TAZ-AXL-CTGF. Finally, we confirmed that TAZ regulates Axl, and plays an important role in clonogenicity and non-adherent growth in vitro and tumor formation in vivo. These data suggest that TAZ could be a therapeutic target for the treatment of colon cancer.

Introduction

The Hippo pathway plays an important role in cell proliferation, organ size control, and cancer development and progression [1,2,3,4]. YAP and TAZ are both transcriptional co-activators that are inhibited by the Hippo pathway [1,2,3,4]. Aberrant inactivation of the Hippo pathway and/or overexpression of TAZ and YAP results in transcriptional activation of their downstream targets [1,2,3,4].

YAP overexpression induces cell proliferation and epithelial mesenchymal transition (EMT), and inhibits apoptosis and contact inhibition [5,6,7]. Transcriptional activation of epidermal growth factor receptor ligand amphiregulin may account for YAP-mediated induction of cell proliferation, especially under serum-depletion [7], while YAP also cooperates with Myc to promote cell proliferation [8]. Recently, YAP has been shown to play a critical role in stem cell biology. It is induced during pluripotent stem cell reprogramming, whilst silencing of YAP reduces the pluripotency of embryonic stem cells [9]. YAP promotes ovarian cancer progression, and high levels of nuclear expression are inversely associated with patient survival [10]. In particular, YAP is associated with clear cell ovarian tumors, an ovarian malignancy subtype with poor prognosis [11]. YAP has also been shown to play an oncogenic role in esophageal squamous cell carcinoma [12]. In liver cancer, microRNA-mediated inhibition of YAP inhibits tumor characteristics including cell proliferation and invasion [13]. Conversely, there are reports showing an opposite, tumor suppressive, role of YAP in promoting p73-mediated apoptosis [14,15]. In breast and head and neck cancers, YAP has been shown to act as a tumor suppressor in certain circumstances [14,16].

TAZ is structurally homologous to YAP, is likewise inhibited by the Hippo pathway, and also promotes EMT-mediated cancer progression [17,18,19]. TAZ regulates mesenchymal stem cell differentiation by modulating Runx2- and PPARgamma-dependent gene expression [20], as well as stem cell self-renewal through controlling localization of Smad [21]. TAZ plays an important role in the progression of breast [22,23] and non-small cell lung
cancer [24,25]. Importantly, TAZ confers cancer stem cell-related traits on breast cancer cells, further highlighting its importance in tumor initiation and progression [26]. TAZ is also overexpressed in papillary thyroid carcinoma [27].

TAZ and YAP have been shown to interact with several transcriptional factors [2,3,4], with the TAZ family of transcriptional factors (TEAD1-4) being the most relevant in cell proliferation and cancer progression [19,28,29]. The X-ray crystal structures of YAP-TEAD complexes have been resolved and the proposed interaction is supported by and consistent with functional analysis [30,31,32], showing that YAP-TEAD complexes activates gene transcription.

YAP expression was observed in colon adenocarcinoma [33,34,35]. It is overexpressed in human colon cancer specimens and overexpression of YAP promotes cell proliferation and survival in colon cancer cells [36]. Recent findings show that knockdown of TAZ results in a decrease in cell proliferation in culture and tumor growth in vivo [26].

Despite evidence suggesting the potential implication of YAP and TAZ in colon cancer progression, their prognostic significance in colorectal cancer is unknown. In this study, we analyzed the mRNA expression of YAP and TAZ, and two of its downstream target genes, AXL and CTGF, in two independent colon cancer patient cohorts comprising 522 patients. We found that TAZ, but not YAP, is a prognostic marker in colon cancer progression. Furthermore, TAZ-AXL-CTGF co-overexpression, which defines both the expression of TAZ and its transcriptional activity on target gene expression, is a novel prognostic indicator, that is independent of tumor grade and stage, for colon cancer patients. The role of TAZ in colon cancer cell proliferation and oncogenesis was validated by functional study.

Materials and Methods

Extraction of clinical and microarray gene expression data from colon cancer patient datasets

Two colon cancer patient datasets, GSE14333 [37] and GSE17538 [38], available in the Gene Expression Omnibus (GEO) Database (http://www.ncbi.nlm.nih.gov/geo) were included in this study. The GEO website has standardized URLs for its individual datasets, e.g. the overall summary information about the microarray dataset GSE14333 can be accessed at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc = GSE14333. For each GEO data series, links are provided at the bottom of the page to the Series Matrix File(s), which contain the expression values for each gene (probeset) and each microarray. The URLs to the Series Matrix File(s) are also standardized. For GSE14333, the URL was ftp://ftp.ncbi.nlm.nih.gov/pub/geo/DATA/SeriesMatrix/GSE14333. The files in gzip format were then unzipped to the tab-delimited text format, which contain detailed information for statistical analysis. The GSE14333 and GSE17538 datasets are the two largest colon cancer patient datasets on the database, and comprise 522 patients, 458 of whose survival data is available in the database. The GSE17538 data series consists of four SubSeries: GSE17536, GSE17537, GSE19072 and GSE19073. GSE19072 and GSE19073 were excluded from this study as they lack clinical data. Microarray gene expression data were retrieved from the data matrices deposited to the GEO database by the original authors. The gene expression levels in GSE14333 and GSE17538 are represented by base-2 logarithm of the MAS5 value and the RNA values, respectively, as adopted by the original authors. R scripting was used to extract the expression values of a small number of genes (probesets) of interest and the clinical data from the data matrices downloaded from GEO.

Demographic and clinical data of the two patient cohorts

Both age and gender of the patients were available demographic data in the two datasets analyzed in the present study. Patients had a median age of 67 years (Range: 26–92 year-old) and 63.5 years (Range: 23–94 year-old) in GSE14333 and GSE17538, respectively. There were 43% and 46% female, respectively, in cohort GSE14333 and GSE17538. The GSE14333 cohort consists of 290 patients, for whom 226 survival data were available. Fifteen, 32, 51 and 21% of patients had stage A, B, C and D tumors, respectively. The survival data were not available for all the patients of stage D. Survival data were available for all 232 patients in the GSE17538 cohort. Twelve, 31, 33 and 24% patients had tumors of AJCC stage I, II, III and IV, respectively, and 8, 78 and 14% of patients had Grade A, B and C tumors respectively.

Correlations of gene expression levels and clinical data

All statistical analyses were performed using SPSS19.0. The associations between expression levels of genes were analyzed by Spearman’s rank test. Expression levels were further divided into high and low levels using median expression level as the cut-off point for Kaplan-Meier survival analysis. Results were compared by log-rank test. Univariate Cox regression analysis was used to correlate the gene expression levels and patient survival and multivariate Cox regression analysis was used to identify independent predictors for patient survival using a backward stepwise approach with an entry limit of p<0.1 and a removal limit of p>0.05. Patients were divided further into 4 groups based on the expression levels of TAZ, AXL and CTGF; the TAZ-AXL-CTGF-low group consisted of patients who expressed all these 3 genes at low levels; the TAZ-AXL-CTGF-intermediate-low group consisted of patients who expressed one of these three genes at a high level; the TAZ-AXL-CTGF-intermediate-high group consisted of patients who expressed two of these three genes at high levels; the TAZ-AXL-CTGF-high group consisted of patients who expressed all three genes at high levels. The survival time of patients stratified by this grouping method were analyzed by Kaplan-Meier analysis and Cox regression as described above.

Identification of TAZ-AXL-CTGF co-expressing genes

Patients were stratified into four groups based on the expression levels of TAZ, AXL and CTGF as described above. The gene expression patterns of patients in TAZ-AXL-CTGF low subgroup and those in the TAZ-AXL-CTGF high subgroup (whose survival was significantly poorer) were compared. Probesets that were differentially expressed between these two subgroups were identified by 2-sample Welch’s T-test. This test was used to avoid the type I error due to unequal variances of the values of probesets between subgroups. Briefly, a Welch’s t test was applied to each probeset corresponding to a certain gene in the data matrix using our own Java application MyStats. P values and the differential expression in fold changes for all the probesets were generated as tab-delimited worksheets of Excel for further analysis. The genes were prioritized by ascending p-values. The top 100 probesets were prioritized in both patient datasets, and the genes common to both datasets were analyzed further.

Identification of potential inhibitory compounds targeting TAZ-AXL-CTGF overexpressing colon cancer (Connectivity Map)

Gene expression connectivity mapping was performed using Statistically Significant Connection’s Map (sscMap) to identify candidate small molecule compounds that may inhibit the
expression of genes that are co-regulated in TAZ-AXL-CTGF co-expressing aggressive colon cancer [39,40,41]. Of the probesets identified to be co-expressed with TAZ-AXL-CTGF, 35 were present on the Affymetrix HG-U133A microarray platform, which was used to generate the microarray database for the Connectivity Map [39]. The compiled gene signature was then fed to the Java application ssMap [41] as a query signature, and its association with the 6000 gene expression profiles generated by treating cancer cells with over 1000 small molecules were compared. The gene signature perturbation procedure, which increases the specificity of the output results, was applied as previously described [42]. All the small molecular compounds, that were negatively associated with the TAZ-AXL-CTGF-GES, were sorted and ranked by their p-value, perturbation stability and standardized connection score. The p-value that was considered significant was set at a stringent threshold (p = 1/1309), ensuring that the results generated by ssMap yield only maximally one expected false positive small molecule over the 1309 small molecules tested in the sccMap [42]. The top 20 small molecules were then entered into the Pubmed (www.pubmed.com) search engine together with colon cancer to identify research articles that have described their effects of the particular molecules on treatment of colon cancer.

Identification of therapeutic targets for colon cancer patients overexpressing TAZ-AXL-CTGF

Patients who co-overexpressed TAZ-AXL-CTGF were stratified into two groups based on their survival statuses. Differential expressions of different probesets between patients in the TAZ-AXL-CTGF-Alive subgroup and those in the TAZ-AXL-CTGF-Deceased subgroup were identified as described above.

Cell culture and retroviral transduction

HCT116 and SW620 cells were obtained from American Type Culture Collection and maintained in F12K/DME medium supplemented with 10% Fetal Bovine Serum (FBS), 10 ug/ml Penicillin/Streptomycin (P/S) (Life technologies, Carlsbad, CA). The amphotropic Phoenix packaging cell line was obtained from the Nolan Laboratory (Stanford University) and maintained in DMEM medium supplemented with 10% FBS, 10 ug/ml P/S, and 100 ug/ml puromycin. Penicillin/Streptomycin (P/S) (Life technologies, Carlsbad, CA). The amphotropic Phoenix packaging cell line was obtained from the Nolan Laboratory (Stanford University) and maintained in DMEM medium supplemented with 10% FBS, 10 ug/ml P/S, and 100 ug/ml puromycin.

Western blot analysis

Immunoblotting was performed as previously described [18] using SuperSignal West Pico (Thermo Scientific, Rockford, IL). The commercial TAZ antibody was obtained from Imgenex (Seattle, WA) while the scramble shRNA construct (5'-CCCTAAGGTTAAGTCGCCCTCG-3'; shScr) was used for control. Stable cell lines were established by selecting the transfected cells in 2 ug/ml puromycin (Sigma Aldrich, St. Louis, MO).

Anchorage-independent soft agar and cloning assays

For the soft agar assay, 5000 cells resuspended in 0.35% (w/v) agarose in culture medium were overlaid on a solidified 0.5% (w/v) agarose in culture medium. The upper layer was allowed to solidify. Medium with puromycin was added the following day and cells were then incubated at 37°C with 5% CO2. Fresh medium with puromycin was supplemented and the colonies formed were stained with 1 mg/ml Thiazolyl Blue Tetrazolium Bromide (Sigma Aldrich) for 4 hours in the incubator at 37°C with 5% CO2. The excess dye was removed by destaining multiple times with water and the number of colonies was determined.

For clonogenic assay, 500 cells were seeded per well in triplicate in 6-well plate. The culture medium was refreshed every week and the cells were fixed with 4% paraformaldehyde in PBS after 2 weeks incubation at 37°C with 5% CO2. The fixed cells were stained with 0.3% crystal violet (Sigma Aldrich) in 20% ethanol overnight. The cells were rinsed with water, dried and colony number was analyzed.

Tumorigenesis in nude mice

Hundred ul of a cell suspension of 1.5×10⁶/ml were inoculated subcutaneously in the left and right hind flanks of four-to-six week-old female nude mice. Tumor development was monitored after 2 weeks. Mice were then euthanized and the tumors were removed for analysis.

Results

TAZ and YAP mRNA expressions positively correlate with mRNA expression of their downstream target genes, AXL and CTGF

Previously, we and others have shown that AXL and CTGF are two important downstream target genes of TAZ and YAP [23,43,44]. In the present study, we investigated whether the mRNA expression levels of the two transcriptional co-activators in the Hippo pathway, TAZ and YAP, correlate with the mRNA expression of AXL and CTGF. In the 290 colon cancer patients from the GSE14333 dataset, TAZ expression was significantly correlated with both AXL (Spearman’s rank test, r = 0.547, p<0.001; Figure 1A) and CTGF (r = 0.543, p<0.001; Figure 1B) expressions. YAP mRNA expression was also positively correlated with AXL (r = 0.154, p = 0.009; Figure 1C) and CTGF (r = 0.141, p = 0.016; Figure 1D) mRNA expression in the same dataset, but to a lesser extent. In 232 colon cancer patients from GSE17538, TAZ mRNA expression was significantly positively correlated with both AXL (r = 0.752, p<0.001; Figure 1E) and CTGF (r = 0.686, p<0.001; Figure 1F) mRNA expressions, while YAP mRNA was also significantly positively correlated with mRNA expression of both genes, again to a lesser extent (AXL: r = 0.343, p<0.001; Figure 1G and CTGF: r = 0.387, p<0.001; Figure 1H).

TAZ, but not YAP, mRNA expression is a predictor for patient survival

In the 226 patients whose survival data were available from the GSE14333 colon cancer patient cohort, a high level of TAZ mRNA expression was significantly correlated with a shorter survival (high level: mean survival = 72.3 months, 95% Confidence Interval (CI) = 63–81 months; low level: mean survival = 129 months, 95% CI = 121–136 months, p<0.001; Figure 2A). By Cox-regression analysis, TAZ mRNA expression was significantly correlated with survival (Hazard Ratio (HR) = 2.251, 95% CI = 1.626–3.116, p<0.001; Figure 2B) in the GSE14333 colon cancer patient cohort. By multivariate analysis (Figure 2C), TAZ mRNA expression (HR = 2.062, 95% CI = 1.472–3.116, p<0.001) and tumor staging (p<0.001) are both independent predictors of survival in the same cohort. Similarly, a high level of TAZ mRNA expression was significantly correlated with shorter patient survival in the GSE17538 colon cancer patient cohort (high level: mean survival = 84 months, 95% CI = 72–96 months; low level: mean survival = 109 months, 95% CI = 97–120 months, p = 0.011; Figure 2B). By Cox-regression, TAZ mRNA expression is a predictor of survival in this cohort (HR = 1.743, 95% CI = 1.177–
2.582, p = 0.006; Figure 2D). Multivariate Cox-regression analysis also showed that TAZ mRNA expression (HR = 1.998, 95% CI = 1.245–3.205, p = 0.004; Figure 2D) was an independent predictor for survival together with stage (p < 0.001) and grade (p = 0.03) of the cancers in this colon cancer patient cohort. On the other hand, YAP mRNA expression did not significantly correlate with patient survival by Kaplan-Meier analysis (GSE14333: p = 0.519; Figure 2E and GSE17538: p = 0.634; Figure 2F) or by Cox-regression analysis (GSE14333: p = 0.673; Figure 2G and GSE17538: p = 0.979; Figure 2H) in either dataset. These results suggest that TAZ mRNA expression is a novel prognostic marker for colon cancer patients, but YAP is not.

Both AXL and CTGF, downstream target genes of TAZ and YAP, are predictors of patient survival

To investigate whether the functional outcome of the transcriptional program mediated by TAZ and YAP confers prognostic value in colon cancer patients, we analyzed whether the mRNA expression of AXL and CTGF, two of the most well defined target genes of TAZ/YAP-TEAD complexes, correlate with survival of patients in the two colon cancer patient cohorts.

High level mRNA expression of AXL correlated with a shorter colon cancer patient survival time in the GSE14333 cohort, although this correlation was not significant [high level: mean survival = 80 months, 95% CI = 71–88 months; low level: mean survival = 114 months, 95% CI = 101–129 months, p = 0.064; Figure 3A]. In the GSE17538 cohort, high level mRNA expression of AXL was significantly correlated with shorter survival [high level: mean survival = 84 months, 95% CI = 72–96 months; low level: mean survival = 104 months, 95% CI = 94–114 months, p = 0.004; Figure 3B]. By Cox-regression analysis, AXL mRNA expression was a predictor of patient survival in both the GSE14333 (HR = 1.839, 95% CI = 1.230–2.748, p = 0.003; Figure 3C) and the GSE17538 (HR = 2.158, 95% CI = 1.141–4.081, p = 0.018; Figure 3D) cohorts. In the GSE14333 cohort, AXL mRNA expression (HR = 1.631, 95% CI = 1.076–2.471, p = 0.021; Figure 3C) was an independent predictor of patient survival with tumor staging (p < 0.001), while in the GSE17538 cohort, AXL mRNA expression (HR = 3.700, 95% CI = 1.665–8.220, p = 0.001; Figure 3D) was an independent predictor of patient survival together with stage (p < 0.001) and grade (p = 0.055) of the cancers.

Similar results were obtained for CTGF. A high level of CTGF mRNA expression was significantly correlated with shorter patient survival in both GSE14333 [high level: mean survival = 87 months, 95% CI = 74–102 months; low level: mean survival = 98 months, 95% CI = 88–108 months, p = 0.012; Figure 3E] and GSE17538 [high level: mean survival = 85 months, 95% CI = 73–
97 months; low level: mean survival = 105 months, 95% CI = 95–114 months, \( p = 0.004 \); Figure 3F) cohorts. Univariate Cox-regression analysis also showed that CTGF mRNA expression was a predictor for patient survival in both cohorts (GSE14333: HR = 1.667, 95% CI = 1.260–2.232, \( p = 0.001 \); Figure 3G and GSE17538: HR = 1.433, 95% CI = 1.126–1.825, \( p = 0.004 \); Figure 3H). Moreover, CTGF mRNA expression is an independent predictor for survival in both cohorts (GSE14333: HR = 1.543, 95% CI = 1.155–2.061, \( p = 0.001 \); Figure 3G and GSE17538: HR = 1.902, 95% CI = 1.385–2.612, \( p = 0.001 \); Figure 3H) together with stage (GSE14333: \( p = 0.001 \) and GSE17538: \( p = 0.004 \)) and grade (GSE17538: \( p = 0.053 \)) of the cancers.

**TAZ, AXL and CTGF can be used in combination to predict colon cancer patient survival**

As described above, expression of TAZ, AXL and CTGF all correlated with colon cancer patient survival. We further investigated whether their expression could be used in combination as a prognostic marker for colon cancer patients. In the GSE14333 cohort, patients whose tumors had low level expression of the three genes had a mean survival time of 117 months (95% CI = 110–123 months), while those whose tumors overexpressed only one of the three genes also had a mean survival of 117 months (95% CI = 101–132 months). Patients whose tumors had a high level expression of two of the three genes had a mean survival time of 65 months (95% CI = 57–74 months), while those whose tumors had a high level expression of all three genes had a mean survival time of 72 months (95% CI = 60–84 months). The survival time of the patients between these four subgroups were significantly different (\( p = 0.001 \); Figure 4A). In Cox-regression analysis, using the subgroup containing patients whose tumors had a low expression of all three genes as a reference group, we found that patients whose tumors overexpressed one (HR = 3.953, 95% CI = 1.070–14.607, \( p = 0.039 \)), two (HR = 6.503, 95% CI = 1.894–22.330, \( p = 0.003 \)) or all the three genes (HR = 7.656, 95% CI = 2.287–25.628, \( p = 0.001 \)) had an increasing risk for disease progression (Figure 4C).

Similar results were obtained in the GSE17538 patient cohort. Patients whose tumors expressed the three genes at low level had a mean survival time of 108 months (95% CI = 97–119 months), those whose tumors had a high level expression of one of the three genes had a mean survival time of 88 months (95% CI = 72–104 months), those whose tumors overexpressed two of the three genes had a mean survival time of 99 months (95% CI = 78–121 months), while those whose tumors overexpressed all three genes had a mean survival time of 77 months (95% CI = 63–91 months). Increasing incidence of overexpression of these three genes resulted in significantly shorter survival in these patients (Kaplan-Meier analysis, \( p = 0.01 \); Figure 4B). Cox-regression analysis using tumors that overexpressed none of the three genes as reference revealed that patients whose tumors overexpressed one (HR = 1.675, 95% CI = 0.75–3.741, \( p = 0.203 \)), two (HR = 1.770, 95% CI = 0.790–3.966, \( p = 0.166 \)) and all of the three genes (HR = 2.807, 95% CI = 1.482–5.317, \( p = 0.002 \)) had an increased risk for disease progression (Figure 4D).

Indeed, patients whose tumors expressed TAZ, AXL and CTGF mRNA at low levels (GSE14333: mean survival = 117 months, 95% CI = 110–123; GSE17538: mean survival = 108 months, 95% CI = 95–121 months) had superior survival compared to those whose tumors overexpressed all the three genes (GSE14333:

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**Figure 3. The associations between AXL or CTGF, and survival in colon cancer patients.** Kaplan-Meier analyses for AXL mRNA expression in (A) GSE14333 and (B) GSE17538 colon cancer patient datasets. Univariate and Multivariate Cox regression analyses for AXL mRNA expression, age, tumor stage and tumor grade in (C) GSE14333 and (D) GSE17538 colon cancer patient datasets. Kaplan-Meier analyses for CTGF mRNA expression in (E) GSE14333 and (F) GSE17538 colon cancer patient datasets. Univariate and Multivariate Cox regression analyses for CTGF mRNA expression, age, tumor stage and tumor grade in (G) GSE14333 and (H) GSE17538 colon cancer patient datasets.

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Figure 4. The associations between TAZ-AXL-CTGF expression and survival in colon cancer patients. Patients were divided into 4 groups according to the number of genes that they overexpressed (expressed at above the Median level) among TAZ, AXL and CTGF. Kaplan-Meier analyses for TAZ-AXL-CTGF mRNA expression in (A) GSE14333 and (B) GSE17538 colon cancer patient datasets. Univariate and Multivariate Cox regression analyses for TAZ-AXL-CTGF mRNA expression, age, tumor stage and tumor grade in (C) GSE14333 and (D) GSE17538 colon cancer patient datasets.

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Table 1. Genes that are co-regulated with TAZ-AXL-CTGF expression in GSE14333 colon cancer patient cohort.

| Gene and phenotype associated with aberrant expression | 0            | 1            | 2            | 3            |
|--------------------------------------------------------|--------------|--------------|--------------|--------------|
|                                                        | Mean | 95% CI | Mean | 95% CI | Mean | 95% CI | Mean | 95% CI |
| **Epithelial-Mesenchymal transition**                  |      |        |      |        |      |        |      |        |
| ACTA2                                                  | 97   | 84–111 | 105  | 95–114 | 120  | 109–132| 147  | 130–164|
| ZEB1                                                   | 76   | 62–90  | 92   | 83–100 | 112  | 102–122| 163  | 141–186|
| ZEB2                                                   | 21   | 19–23  | 28   | 25–30  | 37   | 34–40  |      |        |
| **Migration and Invasion**                             |      |        |      |        |      |        |      |        |
| DDR2                                                   | 95   | 73–117 | 108  | 99–117 | 126  | 113–139| 189  | 161–217|
| FERMT2                                                 | 105  | 69–140 | 120  | 107–132| 153  | 135–171| 246  | 203–288|
| AKT3                                                   | 48   | 43–52  | 56   | 51–60  | 61   | 56–65  | 79   | 72–85  |
| VCAN                                                   | 469  | 386–552| 671  | 578–764| 728  | 636–820| 1275 | 1103–1447|
| **Colon cancer biomarkers**                            |      |        |      |        |      |        |      |        |
| EFEMP2                                                 | 140  | 125–156| 184  | 166–202| 208  | 187–229| 300  | 268–331|
| SULF1                                                  | 687  | 558–816| 1003 | 867–1139| 1126 | 984–1267| 1764 | 1579–1949|
| TAGLN                                                  | 319  | 208–430| 395  | 347–443| 528  | 435–621| 866  | 705–1026|
| **Calcium binding/signaling**                          |      |        |      |        |      |        |      |        |
| FBN1                                                   | 156  | 125–188| 217  | 184–249| 234  | 209–260| 415  | 352–478|
| CALD1                                                  | 84   | 73–96  | 98   | 89–106 | 106  | 98–114 | 156  | 135–177|
| MGP                                                    | 66   | 39–92  | 84   | 60–107 | 106  | 81–130 | 301  | 216–386|
| MYL9                                                   | 358  | 200–515| 419  | 361–477| 613  | 475–752| 1137 | 904–1371|
| **Angiogenesis**                                       |      |        |      |        |      |        |      |        |
| ANTXR1                                                 | 42   | 39–45  | 50   | 46–53  | 51   | 47–54  | 62   | 57–66  |
| SERPINF1                                               | 412  | 340–485| 595  | 529–662| 681  | 594–768| 1038 | 882–1194|
| **Cytoskeleton associated protein**                    |      |        |      |        |      |        |      |        |
| DPYSL3                                                 | 44   | 35–53  | 58   | 51–65  | 59   | 53–65  | 101  | 86–117 |
| PDLIM3                                                 | 38   | 28–47  | 43   | 39–47  | 56   | 50–62  | 86   | 73–100 |
| **Membrane trafficking**                               |      |        |      |        |      |        |      |        |
| RAB31                                                  | 343  | 283–404| 489  | 427–551| 556  | 480–631| 786  | 696–876|
| RAB34                                                  | 88   | 69–107 | 126  | 112–140| 158  | 137–179| 226  | 200–252|
| **Focal adhesion**                                     |      |        |      |        |      |        |      |        |
| TGFβ111                                                | 205  | 162–249| 260  | 230–289| 314  | 273–354| 459  | 397–521|
| TNS1                                                   | 43   | 38–48  | 50   | 46–54  | 61   | 55–66  | 78   | 69–87  |
| **Hippo pathway related genes**                        |      |        |      |        |      |        |      |        |
| AMOTL1                                                 | 18   | 15–22  | 19   | 18–21  | 23   | 20–25  | 30   | 26–33  |
| FRMD6                                                  | 130  | 104–155| 175  | 153–197| 217  | 188–246| 351  | 300–401|
| VGGL3                                                  | 47   | 30–64  | 56   | 49–63  | 71   | 62–80  | 107  | 91–123 |
| **Tissue inhibitor of MMPs**                           |      |        |      |        |      |        |      |        |
| TIMP2                                                  | 279  | 234–323| 443  | 393–492| 486  | 433–538| 698  | 632–763|
| TIMP3                                                  | 264  | 221–307| 352  | 299–404| 417  | 362–471| 563  | 502–624|
| **Others**                                             |      |        |      |        |      |        |      |        |
| CCDC80                                                 | 53   | 31–76  | 65   | 57–73  | 82   | 71–92  | 155  | 128–182|
| COL5A1                                                 | 362  | 309–416| 516  | 447–586| 559  | 488–630| 872  | 753–991|
| GEM                                                    | 361  | 267–455| 474  | 418–531| 534  | 484–583| 945  | 800–1090|
| GLUT80                                                 | 123  | 102–144| 167  | 146–187| 184  | 162–205| 274  | 236–311|
| MSRB3                                                  | 86   | 59–113 | 104  | 94–115 | 129  | 112–146| 196  | 167–225|
| NNMT                                                   | 330  | 265–396| 483  | 414–552| 565  | 473–658| 848  | 731–965|

TAZ in Colorectal Cancer
mean survival = 96, 95% CI = 84–108, p = 0.001; GSE17538: mean survival = 91 months, 95% CI = 81–101 months, p = 0.037; Figure S1).

Neither the age nor sex of the patients was a factor that determined the number of TAZ, AXL and CTGF that overexpressed in either colon cancer patient cohort. TAZ-AXL-CTGF expression was only significantly lower in tumors stage A compared to tumors of other stages (p = 0.022), but were not significantly different between other stages in GSE14333. In GSE17538, TAZ-AXL-CTGF expression was not significantly different between AJCC stages or tumor grades. When the patients were stratified by their stage, we found that TAZ-AXL-CTGF expression was still significantly correlated with poor survival in patients with higher stage tumors. In GSE14333, increasing number of genes overexpressed among TAZ, AXL and CTGF was significantly correlated with a shorter survival (HR = 1.694, 95% CI = 1.075–2.670, p = 0.023) and grade C tumors. Similar results were obtained in GSE17538, in which, TAZ-AXL-CTGF expression was associated with poorer survival in patients with tumors of higher grade and higher stage. TAZ-AXL-CTGF expression was correlated with poorer survival in both grade B (HR = 1.315, 95% CI = 1.026–1.683, p = 0.031) and grade C (HR = 1.671, 95% CI = 1.001–2.790, p = 0.05) tumors, as well as AJCC stage 3 (HR = 1.694, 95% CI = 1.075–2.670, p = 0.023) and stage 4 (HR = 1.415, 95% CI = 1.074–1.866, p = 0.014) tumors.

Identification of potential small molecules that could target the 39 gene signature of TAZ-AXL-CTGF co-expression

Analysis to determine alterations in gene expression following small molecule treatment of cancer cells revealed a total of 257 small molecules that were associated with a gene expression signature significantly correlated with the TAZ-AXL-CTGF gene expression signature (the 25 genes that are presented in bold in Tables 1 and 2 together with TAZ, AXL and CTGF). Of the 257 small molecules, 164 had a 100% perturbation stability and 138 of them are inversely correlated with the TAZ-AXL-CTGF gene expression signature. The results are listed in the supplementary information. The top 20 small molecules were further analyzed through a Pubmed search regarding their effects on treatment of colon cancer. We found that amiloride and tretinoin have yielded 55 and 123 publications, respectively, when coupled with colon cancer in the search engine. Several publications have shown their inhibitory effect on colon cancer growth. Amiloride treatment has been shown to inhibit the growth of colon cancer cells in vitro [45] and in vivo [46]. Importantly, it can sensitize doxorubicin resistant colon cancer cells to treatment with doxorubicin [47], suggesting that amiloride and doxorubicin can be combined to treat doxorubicin resistant colon cancer. Tretinoïn, also known as all-trans retinoic acid, has been shown to inhibit proliferation and anchorage-independent growth of colon cancer cells in vitro [48,49] and in vivo [50], probably through regulating the differentiation state of cancer cells [51].

Identification of potential therapeutic targets for patients overexpressing TAZ-AXL-CTGF

We further investigated which genes could be used to further predict survival in TAZ-AXL-CTGF-high patients; these genes may be potential therapeutic targets specific for this group of survival in the TAZ-AXL-CTGF-high group of patients (data not shown). MGP, DDLME5, TAGLN and ZEB2 were predictors of survival in the TAZ-AXL-CTGF-low group of patients, in the combined colon cancer patient cohort (Figure 5). When the two colon cancer patient datasets were combined, as expected, TAZ-AXL-CTGF mRNA expression levels were associated with patient survival (p = 0.001; Figure 5A). A high level of MGP (p = 0.01; Figure 5B), DDLME5 (p = 0.037; Figure 5C), TAGLN (p = 0.044; Figure 5D) and ZEB2 (p = 0.038; Figure 5E) mRNA expression were correlated with a shorter survival time in the TAZ-AXL-CTGF-low group of patients. These results suggest that these four genes may be used as prognostic markers for patients who express low levels of TAZ, AXL and CTGF mRNA.

Table 1. Cont.

| GSE14333 | Number of TAZ/AXL/CTGF that are overexpressed |
|----------|-----------------------------------------------|
| Gene and phenotype associated with aberrant expression | Relative expression levels |
|          | Mean 95% CI | Mean 95% CI | Mean 95% CI | Mean 95% CI |
| **NXN**  | 170 135–205 | 260 201–320 | 274 234–314 | 360 321–400 |
| **PTRF** | 140 117–163 | 170 157–182 | 189 172–205 | 272 242–303 |
| **SFRP2**| 218 100–337 | 305 208–402 | 404 296–511 | 1142 900–1394 |

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Table 2. Genes that are co-regulated with TAZ-AXL-CTGF expression in GSE17538 colon cancer patient cohort.

| Gene and phenotype associated with aberrant expression | 0 | 1 | 2 | 3 |
|--------------------------------------------------------|---|---|---|---|
| Number of TAZ/AXL/CTGF that are overexpressed | 0 | Mean 95% CI | 1 | Mean 95% CI | 2 | Mean 95% CI | 3 | Mean 95% CI |
| Relative expression levels | | | | | | | | |
| **Epithelial-Mesenchymal transition** | | | | | | | | |
| ACTA2 | 175 | 165–184 | 211 | 198–224 | 240 | 228–252 | 292 | 269–315 |
| ZEB1 | 113 | 106–121 | 152 | 140–165 | 185 | 171–200 | 262 | 234–290 |
| ZEB2 | 56 | 54–59 | 70 | 66–75 | 79 | 75–84 | 97 | 92–101 |
| **Migration and Invasion** | | | | | | | | |
| DDR2 | 203 | 190–216 | 250 | 232–268 | 316 | 293–339 | 440 | 392–487 |
| FERMT2 | 133 | 121–144 | 201 | 179–223 | 259 | 232–286 | 416 | 357–475 |
| AKT3 | 80 | 76–84 | 94 | 89–100 | 109 | 102–116 | 127 | 121–133 |
| VCAN | 584 | 515–652 | 945 | 823–1066 | 1314 | 1126–1503 | 2188 | 1958–2419 |
| **Colon cancer biomarkers** | | | | | | | | |
| EFEMP2 | 243 | 230–256 | 310 | 282–339 | 403 | 364–442 | 513 | 476–549 |
| SULF1 | 726 | 633–818 | 1186 | 1026–1345 | 1897 | 1665–2129 | 2803 | 2578–3029 |
| TAGLN | 670 | 619–722 | 855 | 785–925 | 1215 | 1055–1375 | 1779 | 1562–1996 |
| **Calcium binding/signaling** | | | | | | | | |
| FBN1 | 169 | 152–185 | 263 | 231–295 | 376 | 334–418 | 623 | 544–702 |
| CALD1 | 162 | 153–171 | 201 | 188–214 | 234 | 220–249 | 303 | 277–330 |
| MGP | 101 | 91–110 | 151 | 132–169 | 207 | 179–235 | 381 | 308–454 |
| MYL9 | 448 | 398–498 | 643 | 568–717 | 1010 | 830–1190 | 1678 | 1340–2016 |
| **Angiogenesis** | | | | | | | | |
| ANTXR1 | 187 | 180–194 | 216 | 206–226 | 251 | 240–261 | 290 | 278–302 |
| SERPINF1 | 656 | 584–728 | 1106 | 815–1396 | 1315 | 1150–1480 | 1937 | 1708–2165 |
| **Cytoskeleton associated proteins** | | | | | | | | |
| DPYSL3 | 135 | 125–146 | 186 | 168–204 | 226 | 204–248 | 308 | 280–336 |
| PDLM3 | 68 | 65–71 | 84 | 80–89 | 99 | 94–104 | 139 | 118–160 |
| **Membrane trafficking** | | | | | | | | |
| RAB31 | 501 | 443–559 | 711 | 619–804 | 1021 | 889–1154 | 1549 | 1394–1704 |
| RAB34 | 227 | 208–247 | 313 | 253–372 | 347 | 317–378 | 497 | 452–542 |
| **Focal adhesion** | | | | | | | | |
| TGFBI1 | 388 | 358–417 | 465 | 412–517 | 672 | 599–746 | 880 | 796–964 |
| TNS1 | 157 | 149–165 | 185 | 175–195 | 221 | 208–235 | 256 | 244–268 |
| **Hippo pathway related genes** | | | | | | | | |
| AMOTL1 | 106 | 99–114 | 133 | 123–143 | 147 | 136–157 | 167 | 159–175 |
| FRMD6 | 202 | 179–224 | 309 | 265–353 | 429 | 369–490 | 756 | 659–853 |
| VGLL3 | 45 | 41–50 | 67 | 58–76 | 91 | 79–103 | 153 | 130–176 |
| **Tissue inhibitor of MMPs** | | | | | | | | |
| TIMP2 | 706 | 641–771 | 988 | 882–1094 | 1372 | 1254–1489 | 1814 | 1690–1939 |
| TIMP3 | 485 | 431–539 | 694 | 620–769 | 959 | 838–1080 | 1389 | 1269–1508 |
| **Others** | | | | | | | | |
| CCDC80 | 121 | 112–131 | 151 | 137–165 | 201 | 182–221 | 302 | 262–343 |
| COL5A1 | 501 | 451–552 | 699 | 602–795 | 1066 | 929–1204 | 1525 | 1364–1690 |
| GEM | 365 | 328–401 | 620 | 533–708 | 747 | 648–846 | 1177 | 1049–1305 |
| GLT8D2 | 177 | 159–195 | 246 | 215–276 | 351 | 306–395 | 523 | 467–579 |
| MSR8 | 175 | 165–186 | 222 | 208–237 | 284 | 258–310 | 403 | 359–446 |
| NNMT | 487 | 436–539 | 736 | 651–822 | 1074 | 892–1256 | 1543 | 1385–1701 |
patients whose colon cancers are more aggressive. Therefore, we compared gene expression in this group of patients between those patients who were still living and those who were deceased. The two cohorts’ top 100 differentially expressed genes (based on survival status) were compared, revealing that \( \textit{AN01} \) and \( \textit{SQLE} \) are the two genes commonly differentially expressed between those \( \textit{TAZ-AXL-CTGF} \)-high colon cancer patients who are still alive and those who are deceased. By Kaplan-Meier analysis, we found that these two genes themselves were associated with patient survival in both colon cancer datasets (Figure S2). We then investigated whether the associations between \( \textit{AN01} \) or \( \textit{SQLE} \) and patient survival were specific to patients who overexpressed \( \textit{TAZ, AXL} \) and \( \textit{CTGF} \) (i.e. the \( \textit{TAZ-AXL-CTGF} \)-high subgroup). A low level of \( \textit{AN01} \) expression was significantly (GSE14333, \( p = 0.002 \); Figure 6D and GSE17538, \( p = 0.007 \); Figure 6L) associated with better survival in \( \textit{TAZ-AXL-CTGF} \)-high patients in both colon cancer datasets (GSE1433: high level, mean survival = 59 months, 95% CI = 44–73 months, vs. low level, mean survival = 100 months, 95% CI = 86–114 months; GSE17538: high level, mean survival = 66 months, 95% CI = 51–82 months, vs. low level, mean survival = 107 months, 95% CI = 87–127 months), but was not or less significant in patients with other patterns of expression for \( \textit{TAZ-AXL-CTGF} \) (Figure 6A, B, C, I, J, K).

Similarly, a low level of \( \textit{SQLE} \) expression was significantly (GSE14333, \( p = 0.02 \); Figure 6H and GSE17538, \( p = 0.01 \); Figure 6P) associated with better survival for \( \textit{TAZ-AXL-CTGF} \)-high patients in both colon cancer datasets (GSE1433: high level, mean survival = 55 months, 95% CI = 40–70 months, vs. low level, mean survival = 88 months, 95% CI = 73–103 months; GSE17538: high level, mean survival = 62 months, 95% CI = 45–79 months, vs. low level, mean survival = 86 months, 95% CI = 71–101 months), but was not or less significant in patients with other patterns of expression for \( \textit{TAZ-AXL-CTGF} \) (Figure 6E, F, G, M, N, O). When both \( \textit{AN01} \) and \( \textit{SQLE} \) genes were included in multivariate Cox-regression analysis, we found that mRNA expression of \( \textit{AN01} \), but not \( \textit{SQLE} \), was an independent predictor of patient survival together with both tumor stage and \( \textit{TAZ-AXL-CTGF} \) expression in both colon cancer patient datasets (Tables 3 and 4). These results suggest that \( \textit{AN01} \) and \( \textit{SQLE} \) mRNA expression may determine the aggressiveness of \( \textit{TAZ-AXL-CTGF} \)-high tumors and that \( \textit{AN01} \) mRNA expression could be used in combination with \( \textit{TAZ-AXL-CTGF} \) and tumor stage for better prognostication. Our results also imply that blockade of \( \textit{AN01} \) or \( \textit{SQLE} \) mRNA expression or inhibition of these two proteins in this group of patients may prolong survival.

### Discussion

In the present study, we have shown that TAZ mRNA expression is positively correlated with two of its downstream targets, \( \textit{AXL} \) and \( \textit{CTGF} \), and that TAZ is significantly associated with poor survival of colon cancer patients in two independent colon cancer datasets, comprising 522 patients. Interestingly, the upregulation of \( \textit{AXL} \) and \( \textit{CTGF} \), which reflects the increased transcriptional activity of TAZ-TEAD complexes, can be used in combination with TAZ mRNA expression, for better prognostication in these two independent colon cancer patient datasets. Genes that are co-regulated with \( \textit{TAZ-AXL-CTGF} \) overexpression are involved in several important cellular processes, including cell migration, angiogenesis and calcium signaling, as well as others that have already been described as prognostic markers for colon cancer progression. These genes may be upstream factors or downstream effectors of TAZ and the dysregulated Hippo pathway in colon cancers. Importantly, we also identified two potential therapeutic targets, \( \textit{AN01} \) and \( \textit{SQLE} \), for patients with upregulated \( \textit{TAZ-AXL-CTGF} \) expression; downregulation of either of these two genes may greatly improve the survival of \( \textit{TAZ-AXL-CTGF} \)-high colon cancer patients.

An increase in TAZ mRNA may not necessarily correlate with an increase of its transcriptional activity, due to the fact that TAZ could be post-translationally regulated via cytoplasmic sequestration by 14–3–3 [1]. Possible disparity between TAZ mRNA expression and its transcriptional activity may impair the lone

### Table 2. Cont.

| GSE17538 | Number of TAZ/AXL/CTGF that are overexpressed |
|----------|---------------------------------------------|
| Gene and phenotype associated with aberrant expression | Mean 95% CI | Mean 95% CI | Mean 95% CI | Mean 95% CI |
| | 0 | 1 | 2 | 3 |
| **Relative expression levels** | | | | |
| NNX | 283 | 258–308 | 410 | 348–472 | 499 | 440–559 | 681 | 613–748 |
| PTRF | 264 | 246–282 | 317 | 293–341 | 378 | 344–411 | 564 | 513–615 |
| SFRP2 | 331 | 210–451 | 530 | 383–677 | 1036 | 779–1294 | 2199 | 1763–2634 |

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The role of TAZ in colon cancer progression in vitro and in vivo

To confirm the bioinformatics analysis, we investigated the effect of TAZ knockdown in two colon cancer cell lines, HCT116 and SW620. TAZ knockdown in these two cell lines abolished the expression of TAZ and down-regulated AXL expression (Figure 7A). This result is in line with our finding in human specimens showing that AXL is a downstream target of TAZ. Knockdown of TAZ also resulted in a significant reduction in the number of colonies formed in both clonogenic and non-adherent soft-agar assays in these two cell lines (Figure 7B and C). Importantly, these in vivo results were replicated in the in vivo tumorigenesis assay. Both HCT116-shTAZ and SW620-shTAZ cells formed significantly larger tumors in nude mice compared to HCT116-shScr and SW620-shScr cells, respectively (Figure 7D and 7E, respectively). Our results suggest that TAZ expression is required for a higher cell proliferation, non-adherent growth and tumorigenesis in colon cancer cells, traits that are associated with colon cancer progression.
utilization of TAZ mRNA expression as a prognostic marker. Previously, we have shown that AXL [43] and CTGF [44] are both downstream targets of the Hippo pathway. In the present study, we have analyzed the transcriptional outcome of TAZ-TEAD complexes in colon cancer using AXL or CTGF alone or in combination with TAZ and found that patients who co-overexpressed all three genes had significantly poorer survival compared to those who had other expression patterns. Our results, therefore, strongly suggest that TAZ, AXL and CTGF can be used in combination for prognostification in colon cancer patients.

Several genes that are related to EMT are overexpressed in high TAZ-AXL-CTGF expressing patients (Tables 1 and 2). These include ACTA2 [52], ZEB1 [53,54] and ZEB2 [55,56]. Interestingly, ZEB1 has been shown to be a downstream target of TAZ in retinal pigment epithelial cells [57], suggesting that ZEB1 may act as a downstream effector of TAZ to promote cancer metastasis, while ZEB1 and ZEB2 have also been shown to be prognostic markers in colon cancer [53,56]. Genes that govern migration and invasion were also differentially expressed (Tables 1 and 2). AKT3, which has been shown to contribute oncogenic functions similar to other AKT isoforms [58], was up-regulated in the TAZ-AXL-CTGF-high group of patients. DDR2 [59,60], FERMT2 [61,62,63] and VCAN [64] also play significant roles in cell adhesion and migration and are upregulated in TAZ-AXL-CTGF-high group of patients.
Colon cancer biomarkers were also differentially expressed in these two groups of patients (Tables 1 and 2), namely \textit{EFEMP2}, a serum biomarker for early detection of colon cancer [65] and \textit{SULF1}, a protein important in colon cancer diagnosis [66] and whose serum level is elevated in patients with colon adenomas [67]. Genes that are implicated in angiogenesis were also identified (Tables 1 and 2); both \textit{VCAN} [68,69] and \textit{ANTXR1} [70,71,72] promote angiogenesis for cancer progression. Four genes that are involved in calcium binding or signaling were co-regulated with TAZ-AXL-CTGF in the colon cancer specimens, including \textit{FBN1} [73], \textit{CALD1} [74], \textit{MGP} [75] and \textit{MYL9} [76]. Interestingly, genes that play a role in regulating the Hippo pathway were also co-regulated with TAZ-AXL-CTGF. The angiomotin family members act as tumor suppressors by inhibiting the oncogenic functions of YAP and TAZ [44,77], while \textit{FRMD6} also acts as an antagonist of YAP by activating Hippo pathway kinases [78]. In this study, we found that \textit{AMOLT1} and \textit{FRMD6} are co-overexpressed in TAZ-AXL-CTGF positive tumors, suggesting that \textit{AMOLT1} and \textit{FRMD6} may form a negative regulatory loop with TAZ activation, which requires further investigation \textit{in vitro} in colon cancer cell line models. In addition, \textit{VGLL3}, which has been shown to act as a co-activator of TEAD transcription factors is also upregulated in TAZ-AXL-CTGF-high tumors [79].

Treatments targeting TAZ-AXL-CTGF-high cancers are required due to the aggressive nature of this type of colon cancer. We employed two different analyses to identify potential therapeutic agents and gene targets for this type of cancer. In scMap, we found that amiloride and tretinoin may inhibit the gene expression signature associated with this aggressive type of colon cancer; these molecules have been shown in the literature to provide strong inhibitory effects on colon cancer proliferation \textit{in vitro} and \textit{in vivo}. The other 18 small molecules of the top twenty identified by scMap as listed in the supplementary information have yet to be studied in term of their effect on colon cancer progression driven by the overexpression of TAZ-AXL-CTGF co-overexpression.

\textit{ANO1}, also named DOG1, has been shown to be ubiquitously expressed in gastrointestinal stromal tumors [80] and its overexpression is correlated with development of distant metastases in
head and neck squamous cell carcinoma [81]. Recently, ANO1 was shown to promote tumorigenesis and cancer progression via activating MAPK [82]. In the same study, the authors demonstrated that pharmacological inhibition of ANO1 resulted in cancer cell death, however the role of ANO1 in colon cancer progression has not been examined. In the present study, we found that a low
level expression of ANO1 in the aggressive TAZ-AXL-CTGF-high subgroup of colon cancer patients is associated with prolonged survival (high level to low level of ANO1 expression; mean survival from 59 to 100 months and from 66 to 107 month in GSE14333 and GSE17538 patient datasets, respectively), suggesting that pharmacological inhibition of ANO1 may represent a novel therapeutic approach for this group of patients with aggressive colon cancer. ANO1 is a calcium ion-activated chloride channel. Interestingly, multiple genes involved in calcium signaling (FBN1, CALD1, MGP and MYL9) were co-overexpressed with TAZ-AXL-CTGF in colon cancer. Due to the fact that calcium signaling plays an important role in cancer progression [83,84,85], further investigation into the relationship between ANO1 and calcium signaling in TAZ-AXL-CTGF-mediated cancer progression is warranted. Nonetheless, our results provide a clue to the involvement of calcium signaling and Ca$^{2+}$-activated Cl$^{-}$ channels in colon cancer progression mediated by the Hippo pathway.

Little is known about how SQLE promotes cancer progression. It has been shown to be overexpressed in lung squamous cell carcinoma by suppression subtractive hydridization [86], in pancreatic cancer by genome-wide analysis using microarray based techniques [87], and in prostate cancer progression by bioinformatics analysis [88]. Its overexpression in breast cancer is correlated with decreased distant metastasis-free survival [89]. These results show that SQLE promotes cancer progression in multiple types of cancer. Again, its role in colon cancer progression was undefined. In the present study, we also found that a high level expression of SQLE is associated with poorer survival in TAZ-AXL-CTGF-high patients to be associated with poorer survival (high level to low level of SQLE expression; mean survival from 55 to 88 months and from 62 to 102 month in GSE14333 and GSE17538 patient datasets, respectively), suggesting that SQLE may be a novel therapeutic target for this group of patients.

In conclusion, this study has shown that TAZ-AXL-CTGF in combination may be a novel prognostic indicator for colon cancer progression, and that their overexpression is associated with increased expression of genes that are associated with colon cancer progression. Furthermore, ANO1 and SQLE overexpression may further define a poorer prognosis for colon cancer patients overexpressing TAZ-AXL-CTGF.

### Table 3. Cox-regression analysis of GSE14333 datasets.

| Clinicopathological variables | Multivariate analysis (Forward conditional) |
|------------------------------|-------------------------------------------|
|                              | Hazard ratio (95% CI) | p-value |
| Stage (n = 226)              | Overall               | 0.001   |
| A (n = 41)                   | 1                      | Reference |
| B (n = 94)                   | 2.648 (0.592–11.843)  | 0.203   |
| C (n = 91)                   | 7.213 (1.688–30.817)  | 0.008   |
| Hippo pathway activity (n = 226) | 0.086                |         |
| 0 gene overexpressed (n = 59) | 1                      | Reference |
| 1 gene overexpressed (n = 52) | 3.798 (1.009–14.302) | 0.049   |
| 2 genes overexpressed (n = 54) | 4.961 (1.428–17.236) | 0.012   |
| 3 genes overexpressed (n = 61) | 4.505 (1.310–15.492) | 0.017   |
| ANO1 (n = 226)              | 1.550 (1.161–2.069)  | 0.003   |

### Table 4. Cox-regression analysis of GSE17538 datasets.

| Clinicopathological variables | Multivariate analysis (Forward conditional) |
|------------------------------|-------------------------------------------|
|                              | Hazard ratio (95% CI) | p-value |
| Age (n = 213)                | Overall               | 0.029   |
| Stage (n = 213)              |                         | 0.001   |
| 1 (n = 27)                   | 1                      | Reference |
| 2 (n = 65)                   | 4.089 (0.513–32.586)  | 0.184   |
| 3 (n = 70)                   | 6.898 (0.887–53.641)  | 0.065   |
| 4 (n = 51)                   | 79.966 (10.374–616.406) | 0.001   |
| Hippo pathway activity (n = 213) | 0.013                |         |
| 0 gene overexpressed (n = 64) | 1                      | Reference |
| 1 gene overexpressed (n = 38) | 1.106 (0.439–2.787)  | 0.830   |
| 2 genes overexpressed (n = 34) | 1.050 (0.410–2.691) | 0.920   |
| 3 genes overexpressed (n = 77) | 2.784 (1.208–6.419) | 0.016   |
| ANO1 (n = 213)              | 1.855 (1.286–2.677)  | 0.001   |

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Supporting Information

Figure S1  Colon cancer patients expressing low levels of TAZ, AXL and CTGF had superior survival. Patients were stratified into two groups; those whose tumors expressed TAZ, AXL and CTGF mRNA at low level (solid line) and those whose tumors expressed at least one of TAZ, AXL and CTGF at high level (dotted line). Kaplan-Meier analyses for these two subgroups of patients in (A) GSE14333 and (B) GSE17538 colon cancer datasets.

Figure S2 The associations between ANO1 or SQLE, and survival in colon cancer patients. Kaplan-Meier analyses for (A) ANO1 and (B) SQLE mRNA expression in the GSE14333 colon cancer patient dataset. Kaplan-Meier analyses for (C) ANO1 and (D) SQLE mRNA expression in the GSE17538 colon cancer patient dataset.

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