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Nuclear expression of Gli-1 is predictive of pathologic complete response to chemoradiation in trimodality treated oesophageal cancer patients

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Background: Predictive biomarkers or signature(s) for oesophageal cancer (OC) patients undergoing preoperative therapy could help administration of effective therapy, avoidance of ineffective ones, and establishment new strategies. Since the hedgehog pathway is often upregulated in OC, we examined its transcriptional factor, Gli-1, which confers therapy resistance, we wanted to assess Gli-1 as a predictive biomarker for chemoradiation response and validate it.

Methods: Untreated OC tissues from patients who underwent chemoradiation and surgery were assessed for nuclear Gli-1 by immunohistochemistry and labelling indices (LIs) were correlated with pathologic complete response (pathCR) or $<$pathCR (resistance) and validated in a unique cohort.

Results: Initial 60 patients formed the discovery set (TDS) and then unique 167 patients formed the validation set (TVS). 16 (27%) patients in TDS and 40 (24%) patients in TVS achieved a pathCR. Nuclear Gli-1 LIs were highly associated with pathCR based on the fitted logistic regression models ($P<0.0001$) in TDS and TVS. The areas under the curve (AUCs) for receiver-operating characteristics (ROCs) based on a fitted model were 0.813 (fivefold cross validation (0.813) and bootstrap resampling (0.816) for TDS and 0.902 (fivefold cross validation (0.901) and bootstrap resampling (0.902)) for TVS. Our preclinical (including genetic knockdown) studies with FU or radiation resistant cell lines demonstrated that Gli-1 indeed mediates therapy resistance in OC.

Conclusions: Our validated data in OC show that nuclear Gli-1 LIs are predictive of pathCR after chemoradiation with desirable sensitivity and specificity.

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The incidence of oesophageal cancer (OC), particularly adenocarcinoma (EAC), has risen in recent decades. In 2015, the estimated number of new cases and deaths due to OC in the United States (US) are 16 980 and 15 590, respectively (American Cancer Society (2015)). In the US, standard approach for localised OC (LOC) is chemoradiation followed by surgery (when feasible) (van Hagen *et al*, 2012; Ajani *et al*, 2015a). Approximately 25% of patients' OCs are highly sensitive to chemoradiation and result in a pathologic complete response (pathCR, defined as no cancer cells in the resected specimen). PathCR patients, generally, have a better outcome than those patients whose OCs achieve <pathCR (Berger *et al*, 2005; Chirieac *et al*, 2005; Rohatgi *et al*, 2005a, b; Rizk *et al*, 2007; Donahue *et al*, 2009; Cheedella *et al*, 2013). However, for a given patient, the degree of response and the prognosis are frustratingly unpredictable. Validated reliable clinical variables (Ajani *et al*, 2012) or biomarker(s) are currently unavailable. We have previously reported that ALDH1 in OC tissues appears to be related to resistance to chemoradiation (Ajani *et al*, 2014), however, further research with additional biomarkers is necessary prior to clinical implementation and the biomarker(s) or signature(s) must accommodate inherent heterogeneity of OC.

One potential advantage of this research trajectory, although this concept remains theoretical, is to identify a patient whose OCs may have extreme results in the response spectrum. For example, identification of patients whose OC are destined to achieve a pathCR could be the subjects of an oesophageal preservation strategy and those with extremely resistant OC could move directly to surgery (bypassing chemoradiation) until we have tools to overcome resistance. We have previously reported that the hedgehog (HH) pathway is dysregulated in OC, it mediates resistance to therapy, and inhibition of the HH pathway overcomes resistance to cytotoxics and radiation (Sims-Mourtada *et al*, 2006, 2007). We also demonstrated that Gli-1 can participate in a crosstalk with mTOR pathway to induce secondary resistance to HH inhibition in OC (Wang *et al*, 2012). The HH pathway and associated overexpression of Gli-1 has been reported by others as oncogenic (Onishi and Katano, 2011), including in basal cell carcinomas (Rudin *et al*, 2009; Von Hoff *et al*, 2009), small cell lung cancer (Park *et al*, 2011), medulloblastomas (Pasca di Magliano and Hebrok, 2003), breast cancer (Souzaki *et al*, 2011), prostate cancer (Karhadkar *et al*, 2004), and pancreas cancer (Nagai *et al*, 2008; He *et al*, 2011). In squamous cell OC, the Gli-1 nuclear expression was reported as an independent variable for relapse and poor prognosis (Yoshikawa *et al*, 2008). Gli-1 signalling is intertwined with cancer stem cell (CSC) maintenance (Po *et al*, 2010; Coni *et al*, 2013; Ajani *et al*, 2015b). Gli-1 has been amply implicated in resistance to therapy through the mechanism of inducible glucuronidation (Zahreddine *et al*, 2014). In glioma patients, a positive correlation was observed between Gli-1 expression and tumour recurrence (Cui *et al*, 2010). Based on our research and the contributions by others, we hypothesised that the expression of nuclear Gli-1 could be predictive of response, particularly pathCR, to chemoradiation in OC patients. We are more interested in discovering a predictive biomarker or signature because it is more likely to help change clinical practice rather than a prognostic biomarker (where the chance of its being a therapeutic target could be low). Gli-1 as a predictive biomarker for OC (or in any other tumour type) has not been reported, to our knowledge.

MATERIALS AND METHODS

Patient population and therapy. Through an institutional review board (IRB) approved protocol and projects supported by the National Cancer Institute and UT M. D. Anderson Cancer Center (MDACC), pre-treatment cancer specimens were obtained from 227 patients who met the following criteria: had localised (T1N1, T2, T3 with any N or with M1a), histologically confirmed adenocarcinoma or squamous cell carcinoma of the thoracic esophagus, and were treated chemoradiation followed by surgery. All patient material was collected after obtaining an informed written consent. Following institutional standards, each patient underwent complete baseline clinical staging and was discussed in the weekly Oesophageal Cancer Conference prior to the initiation of therapy. Positron emission tomography was allowed when feasible. Chemotherapy included a fluoropyrimidine with either a platinum compound or taxane with concurrent 50.4 Gray radiation in 28 fractions. Surgery was performed ~6–7 weeks after the completion of chemoradiation. The surgical specimens were scored by the method described by (Chirieac *et al*, 2005). This method has been independently validated in a multi-institutional setting (Wu *et al*, 2007). Specimens were scored by two team members (independently) in a blinded manner to prevent the potential bias. Each specimen was designated as pathCR or <pathCR (extreme resistance defined as >50% residual OC was also assessed).

All patients were followed for 5 years or until death as described previously (Ajani *et al*, 2014).

Statistical methods. Patient characteristics were summarised using median (range) for continuous variables and frequency (percentage) for categorical variables. Overall survival (OS) was defined as the time interval between surgery and the date of death due to any cause. Patients who were alive were censored at the last follow-up date. Progression-free survival (PFS) was defined as the time interval between surgery and the date of relapse or death due to any cause. Patients who were alive and relapse-free were censored at the last follow-up date. The probabilities of OS and PFS were estimated using the method of Kaplan and Meier (Kaplan and Meier, 1958). Log-rank test (Mantel, 1996) was used to assess the difference in OS or PFS between subgroups of patients. Univariate logistic regression model was fit to assess the association between Gli-1 and the probability of achieving pathCR. Based on fitted model, a plot of Gli-1 LI (%) *vs* the predicted probability of pathCR was created. The receiver operating characteristics (ROC) curve was also generated to derive the area under the curve (AUC) and to assess the overall predictive ability of the fitted model. Two resampling techniques (cross validation and bootstrapping) were used to validate the estimated AUC. The sensitivity, specificity, positive predictive value, negative predictive value and predictive accuracy for pathCR based on various cutoff values of Gli-1 are also summarised. All statistical analyses were performed with SAS and Splus software.

Preclinical methods

Cell lines and reagents. The human EAC cell lines SKGT4 (SK4) and Flo-1 were acquired from our institution and described previously (Soldes *et al*, 1999; Raju *et al*, 2006). To establish 5-FU-resistant subclones, SKGT4-RF (SK4-RF), SK-4 parenteral cells were treated at their IC50 concentration of 5-FU for 3–5 weeks, and then the concentration of 5-FU was increased every two to three weeks until the resistant clones were established. This procedure was repeated four times. The establishment of these 5-FU-resistant subclones took 3–6 months and newly derived 5-FU-resistant clones were designated SK4-RF. Similarly; Flo-1 cells were made resistant to radiation and termed Flo-1 XTR. To establish radiation resistant subclones, Flo-1 parental clones were irradiated to 2 Gy and maintained in culture. The surviving clones were allowed to achieve 80% confluency (~1–2 weeks) and again irradiated to 2 Gy. This process was performed a total of four times. Once complete, cells were stored at −80 °C until use. Cells were kept in continuous culture for less than 2–3 weeks, with subsequent experiments using fresh lots of cells. These cells were authenticated and re-characterised in the core facility of MDACC

every 6 months. Cells were cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics (100 mg/ml streptomycin and 100 IU/ml of penicillin; Ajani *et al*, 2014) and incubated at 37 °C in 5% CO2. 5-FU was purchased from Sigma Chemical Co. (St Louis, MO, USA).

Gli-1 inhibitor was purchase from Selleck Chemical LLC (Houston, TX, USA) and reconstituted in 100% ethanol. The Lenticrispr Gli1 was constructed in our lab using the CRISPRs (clustered regularly interspaced short palindromic repeats)/Cas9 system. Guide RNAs design follows MIT Feng Zhang's website http://crispr.mit.edu/ or German Cancer Research Center's E-Crisp website http://www.e-crisp.org/E-CRISP/designcrispr.html. Human Gli1 gene is designed with 2 targets of guide RNA sequences as below.

hGli1.E5.gRNA3.F 5′-caccgGGGAAGGGTCCCCGGGACTG-3′
hGli1.E5.gRNA3.R 5′-aaacCAGTCCCGGGGACCCTTCCCc-3′
hGli1.E5.gRNA4.F 5′-caccgAGGAAGGCGAGGGCCCTTTT-3′
hGli1.E5.gRNA4.R 5′-aaacAAAAGGGCCCTCGCCTTCCTc-3′

With 100 μM 50 μl of each primer added together, each pair of guide RNA sequences is formed into duplexes in a heating block heated to 100 C and cooled gradually by itself. The duplexes are then used as inserts ligated using T4 DNA ligase(NEB, Ipswich, MA, USA) into LGP (lentiGuidePuro, Addgene) cut by BsmbI (NEB). The ligates are transformed into Stabl3 competent cells, resultant clones are screened by the sises of inserts and verified by sequencing.

LGP plasmids with right inserts of Gli1gRNAs are then co-transfected with pLenti-Cas9-Blast, pCMV.Dr8.2 and pCMV-VSV.G with ratio of 10 : 10 : 10 : 1 into HEK293T cells with ~70% confluency by Lipofectamine2000 (Carlsbad, CA, USA) in six-well plates. Supernatants are collected 48 h later and recollected 24 h later for second time. Lentiviral supernatants are centrifuged and clear upper solutions are used for immediate transduction or kept in −80 °C freezer for later use.

Target cell line Flo1-XTR is seeded in six-well plates with ~70% confluency, lentiviral supernatants are added with 8 mM polybrene, transduced cells are then selected by puromycin at concentration determined by kill curves for 4–6 days. Surviving cells are then expanded and positive pools are screened by western blot of appropriate antibodies.

Tissue specimens and handling. Untreated OC biopsies were used as described previously (Ajani *et al*, 2014). OC histology and ⩾50% tumour cellularity were confirmed before staining for Gli-1. The tissue sections were 4 μ thick. Immunohistochemistry staining for Gli-1 were performed using anti-Gli-1 ab92611 (1 : 400) antibody. Positive and negative controls were used previously reported (Sims-Mourtada *et al*, 2006). Two team members independently reviewed the slides to establish Gli-1 LIs, on the basis of percentage of tumour cell nuclei stained and the staining intensity. Joint consensus was made for discordant cases using a double-headed microscope for re-review.

Cell proliferation assay. Cell proliferation on SK4 and Flo-1 OC cells and their resistant counterpart SK4-RF and Flo-1 XTR were performed using the CellTiter 96 aqueous nonradioactive cell proliferation assay (MTS) according to the instructions of the manufacturer (Promega Co., Madison, WI, USA) as described as before (Song *et al*, 2015).

Clonogenicity assay. Single cells (800/well) were seeded in triplicates onto a six-well plate (Falcone). The cell culture medium and incubation condition have been described in the Cell lines and reagents. Twenty-four hours later, cells were either treated with GANT61 (10 μM) or same amount 100% ethanol as negative control. Nine days after seeding, the cells were washed and then fixed with 3.7% paraformaldehyde for 20 min. Subsequently, the cells were washed twice in tap water and stained with 0.3% crystal violet for 2 min at room temperature. Following washing with tap water, colonies were counted by eye.

Tumour sphere formation assay. Single cells (800/well) were seeded in triplicate onto a 24-well ultra-low attachment plate (Corning) in serum free DMEM/F-12 supplemented with 10 ng epidermal growth factor, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, and bovine pituitary extract (Invitrogen). After 12 days of culture under the same condition as described in the Cell lines and reagents, tumour spheres formed and their number was counted under a microscope.

Matrigel invasion assay. The invasive capacity of cells was studied by using invasion chambers with 0.8 uM pore sise (Greiner bio-one) inserted into a 24-well plate (Falcone). A signal cell suspension containing 2.5×10^4 was added into the invasion chamber. After 24 h of incubation in the same environment described in the Cell lines and reagents, cells on the upper surface of the invasion chamber were removed with cotton swabs. Invaded cells which adhered on the lower surface of the membrane were fixed and stained with Diff-Quik (Siemens), then photographed under a microscope and counted.

Protein extraction and western blot analysis. Protein isolation and Western blot analyses were performed in Sk4/Sk4-RF and Flo-1/Flo-1 XTR OC cells as previously described (Song *et al*, 2014) and immunoreactive bands were visualised by chemiluminescence detection.

Reverse-phase protein arrays. The reverse-phase protein arrays (RPPA) analysis was performed in Sk4 cells and its resistant clone Sk4-FR cell lysate in the RPPA core facility of MDACC. Samples were serially diluted 2-fold for 5 dilutions and probed with 175 antibodies and arrayed on nitrocellulose-coated slides. The relative protein levels were normalised for protein loading and determined by interpolation of each dilution curve from the standard curve as previously described (Hennessy *et al*, 2010). Gene set enriched analysis (GSEA) conducted by a bioinformatician (Dr Bin Liu).

Real-time quantitative reverse transcription PCR. Total RNAs from cell cultures are extracted by using Trizol (Ambion, Austin, TX, USA) concentrations of RNAs are measured by Nanodrop 1000 (Nanodrop, Wilmington, DE). First strand cDNAs are synthesised by Reverse transcription PCR using Invitrogen's Superscript III kit (Invitrogen, Carlsbad, CA, USA). Quantitative RT PCR measuring mRNA expression levels are performed by ABI 7500 (Life Technologies, Grand Island, NY, USA) using the following listed primers. The fluorescence threshold cycle (Ct) value was determined for each gene and normalised with GAPDH. Relative quantitation is calculated by using $RE = 2^{(-\Delta\Delta Ct)}$. The primers used as followings: Reference primers: hGAPDH 5′-ACCCAGAAGACTGTGGATGG-3′, hGAPDH-3 5′-TCTA-GACGGCAGGTCAGGTC-3′;

Target genes' primers are:
hGli1.mRNA.F 5′-CCAGCGCCCAGACAGAG-3′
hGli1.mRNA.R 5′-ACAGTCCTTCTGTCCCCACA-3′
hGli2.mRNA.F 5′-CTCTCCTTTGGTGGTGGCT-3′
hGli2.mRNA.R 5′-GGTGTGTGTCCAAAGGCTG-3′;
hSHH.mRNA.F 5′-CCAATTACAACCCCGACATC-3′
hSHH.mRNA.R 5′-AGTTTCACTCCTGGCCACTG-3′

RESULTS

Patients, chemoradiation response, and survival. Initial 60 patients formed the discovery set (TDS) and subsequent 167 patients formed the validation set (TVS). The group prior to proceeding with TVS reviewed results on TDS set. Patient

characteristics of TDS and TVS are summarised in Table 1. For TDS, the median follow up time was 44.4 months and median OS was 54.6 months (95% CI: 34.1 to not estimable). In TDS, 31 patients have died and the median PFS was 30.3 months (95% CI: 17 to not estimable). In TVS, the median follow up time of all patients was 81.5 months and the median OS was 41.8 months (95% CI: 28.2 -53.3 months). The PFS was 21.6 months (95% CI: 14.4–38.4 months). Sixteen (27%) patients in TDS and 40 (24%) patients in TVS had a pathCR and the rest had <pathCR. The median OS of pathCR patients was longer than that of those who's OCs achieved a <pathCR (TDS, median OS not reached in pCR patients *vs* median OS of 34.1 months in <pCR patients, $P=0.005$; TVS, median OS of 60 months in pCR patients *vs* 36.9 months in <pCR patients, $P=0.10$).

Gli-1 expression and correlation with response. The median nuclear Gli-1 LI was 20% (range, 0–95%). On the basis of the fitted the logistic regression model, Gli-1 was significantly associated with the probability of achieving a pathCR in TDS (Table 2) and in TVS (Table 3). Patients with a higher Gli-1 LIs had a lower probability of pathCR (TDS, OR [odds ratio] = 0.46; 95% CI 0.33–0.64; $P=<0.0001$. TVS, OR = 0.84; 95% CI: 0.78–0.90; *P* value <0.0001). Figure 1A and B show that most pathCR patients gravitated towards lower nuclear Gli-1 LIs and the resistant population towards higher LIs.

On the basis of the fitted logistic regression model, the AUC of the ROC (Figure 2A and B) for Gli-1 was 0.813 (fivefold cross validation (0.813) and bootstrap resampling (0.816) for TDS and 0.902 (fivefold cross validation (0.901) and bootstrap resampling (0.902)) for TVS. Supplementary Tables 1 and 2 show the estimated sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) and the overall predictive accuracy of pathCR based on choosing different cutoff values for Gli-1. The results demonstrate a relatively high specificity (>85%) for lower nuclear Gli-1 LIs (⩽10%).

Chemo/radiation-activated Gli-1 and Shh expression. Gli-1 and Shh are important hedgehog pathway (Hh) signalling components and are associated with therapy response/resistance. We observed that Gli-1 and its ligand Shh have increased expression in EAC tumour tissues compared to Barrett's premalignant tissues (Supplementary Figure S1) and we found nuclear expression of Gli-1 was significantly associated therapy response. Thus, the mechanism underlying the effect of chemo/radiation on the Hh signalling was investigated. It was observed that 5-FU or radiation induced resistant cells SK4-RF and Flo-1 XTR had higher expression of nuclear Gli-1 and Shh (Figure 3A). Increased mRNA levels of Gli-1, Gli-2 and Shh in chemoresistant cells, SK4-RF were confirmed using quantitative real-time PCR (Figure 3B). RPPA further demonstrated that many oncogenes were enriched in therapy resistant cells (SK4-RF) compared to the parental cells (Figure 3C). These data indicate that chemo/radiation therapy activates the Hh signalling which may mediate the therapy response/resistance.

Table 1. Patient characteristics

Covariate	Levels	Discovery set *N* (%)	Validation set *N* (%)
Age (years)	Median	59	62
	Range	35–76	27–80
Gender	Male	59 (98.33)	149 (89.22)
	Female	1 (1.67)	18 (10.78)
Ethnicity	White	58 (96.67)	152 (91.02)
	Hispanic	1 (1.67)	13 (7.78)
	African American	1 (1.67)	2 (1.2)
Clinical stage[a]	IIA	24 (40.00)	59 (35.33)
	IIB	3 (5.00)	7 (4.19)
	III	30 (50.00)	81 (48.5)
	IVA	2 (3.33)	9 (5.39)
	IVB	1 (1.67)	4 (2.40)
	X	0 (0.00)	7 (4.19)

[a]AJCC 6th edition.

Table 2. Logistic regression model for pathCR in TDS (*n* = 60; pathCR = 16)

Variable	OR	95% CI	*P*-value
Intercept	–	–	<0.0001
Gli-1*100	0.46	0.33–0.64	<0.0001

Abbreviations: OR = odds ratio; pathCR = pathologic complete response; TDS = the discovery set.

Table 3. Logistic regression model for pathCR in TVS (*n* = 167; pathCR = 40)

Variable	OR	95% CI	*P*-value
Intercept	–	–	0.003
Gli-1*100	0.84	0.78–0.90	<0.0001

Abbreviations: OR = odds ration; pathCR = pathologic complete response; TVS = the validation set.

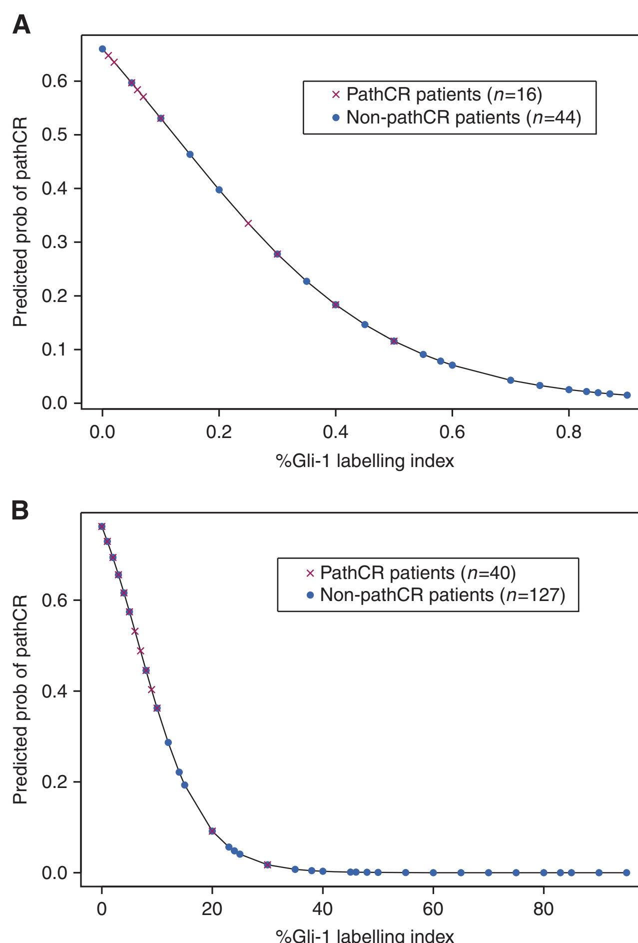

Figure 1. (**A**) Plot of % Gli-1 LI *vs* the predicted probability of pathCR based on the fitted model in Table 2 for TDS (the discovery set). (**B**) Plot of % Gli-1 LI *vs* the predicted probability of pathCR based on the fitted model in Table 3 for TVS (the validation set).

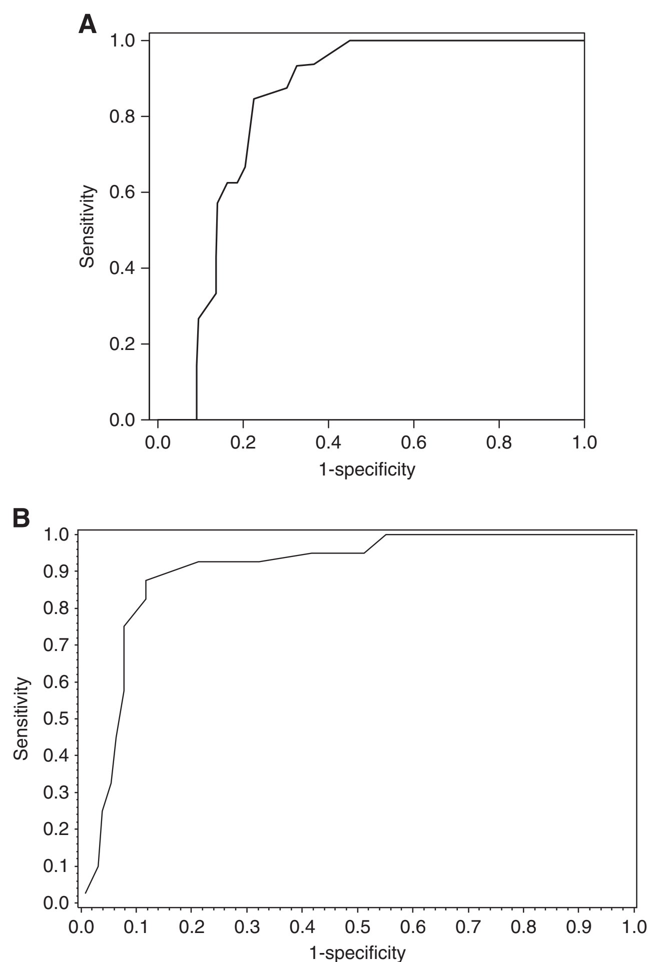

Figure 2. (**A**) ROC AUC indicating sensitivity and specificity based on the fitted model in Supplementary Table S1 for TDS (the discovery set; $n=60$). (**B**) ROC AUC indicating sensitivity and specificity based on the fitted model in Supplementary Table S2 for TVS (the validation set; $n=167$).

Chemo/irradiation endows EAC cells with high potentials of cell proliferation, clonogenecity, and tumour sphere formation *in vitro* in OC cells. After showing that chemo/radiation resistant cells over-expressed Gli-1 and Shh, we sought to determine if the resistant cells conferred more malignant behaviour. As expected, we found that both chemo (SK4-RF) and radiation resistant cells (Flo-1 XTR) had higher rates of proliferation compared to their parental counterparts (Figure 3D). Clonogenicity has also been employed as a metric of resistance to radiation and chemotherapy. Our colony-genicity assay further confirmed that radiation resistant Flo-1 XTR cells dramatically increased colony formation (Figure 3E). The formation of tumour spheres has been considered as a surrogate indicator of CSC properties in epithelial cancers (Dontu *et al*, 2003). We analysed the tumour sphere formation in Flo-1 XTR as well as in parental cell line Flo-1. The irradiation survived cells Flo-1 XTR proliferated and generated larger tumour spheres, while parental Flo-1 cells did not form any tumour spheres (Figure 3F, left panel). The number of tumour spheres developed in the radiation resistant cells (Flo-1 XTR) was significantly higher and larger than that of parental cell line Flo-1 (Figure 3F, right panel). This indicates that irradiation treatment endows CSC properties to OC cells.

Higher proliferation and tumour sphere formation rates are correlated with higher expression of Gli-1. The chemo (SK4-RF) and radiation (Flo-1 XTR) resistant cell lines showed higher proliferation and tumour sphere formation rates than the parental cell lines. To investigate the cause-effect relationship, we used lentCrisp/cas9 system (Supplementary Figure S2A and B) and GANT61 (Supplementary Figure S2C and D), a specific Gli-1 inhibitor to genetically knockdown *Gli-1* expression or pharmacologically block the Gli-1 signalling pathway respectively. We found that both (genetic knockdown of *Gli-1* and pharmacologic inhibition of Gli-1 protein) significantly decreased cell proliferation and sensitised cells to radiation (Supplementary Figure S2B and D). Also, tumour sphere formation (Supplementary Figure S2A) was dramatically reduced by lentCrisp/cas9 system, while cell invasion (Supplementary Figure S2C) was decreased as well.

DISCUSSION

The research portfolio for patients with localised OC has generally been limited to empiric clinical trials to improve the outcome of patients. In this regard, some advances have been realised (Cooper *et al*, 1999; Wu *et al*, 2007; van Hagen *et al*, 2012; Ajani *et al*, 2015a). However, chemoradiation and surgery are associated with considerable morbidity and surgery particularly results in life-altering consequences. The current approach that emphasises baseline clinical staging and stage grouping in order to make initial and long-term therapy decisions do not account for inherent molecular heterogeneity of OC. Thus some patients seem to benefit and others do not but at the outset one has no idea what therapy is optimum for a given patient. In addition to not being able to select an effective therapy for a given patient, we also have little knowledge of molecular biology of OC. Recent effort by The Cancer Genome Atlas (TCGA) has demonstrated stark biology difference between squamous cell carcinoma and adenocarcinoma, however, several subgroups (with different genomic makeups) have also been described. (Cancer Genome Atlas Research N *et al*, 2017) TCGA analysis provides impetus for further exploration before such platforms can provide clinical guidance. Therefore, our general knowledge needs to considerably expand. A glaring example is that EGFR is overexpressed in squamous and adenocarcinoma of the esophagus and is prognostic (Wang *et al*, 2007); however, the assumption that these tumours are primarily driven by EGFR was incorrect as demonstrated by several clinical trials that attempted inhibition of the EGFR pathway by various means but failed miserably (Chan *et al*, 2011; Crosby *et al*, 2013; Lordick *et al*, 2013; Waddell *et al*, 2013). We have shown that Yap1 upregulates EGFR at the transcription level and therefore, inhibition of Yap1 lowers the expression of EGFR and reduces cell survival (Song *et al*, 2015). However, Yap1 inhibitors have not yet been tried against OC and not available in the oncology space.

Our quest has been to identify predictive biomarkers to individualise therapy in patients with OC. Our preclinical data suggest that CSCs seem to play a major role in mediating resistance to therapy in OC. Like ALDH-1 (Ajani *et al*, 2014), Gli-1 (in the Hh pathway) is related to CSC maintenance. The preclinical data presented in this report suggest that when sensitive cells are made resistant to cytotoxic drug or radiation, the Hh pathway (particularly, Gli-1) is upregulated. Using modern CRISPR/Cas9 technology and prior established clonogenic assay, we document in the preclinical setting that Gli-1 activates resistance to therapy (chemotherapy as well as radiation). The clinical data are compelling. Nuclear Gli-1 LIs highly correlated with response to chemoradiation. Of great interest here is the tight correlation with pathCR and data are validated in a large independent cohort. We acknowledge that considerably more work is needed before predictive biomarkers can be clinically implemented. However, we believe these compelling results represent early steps in the development of personalised medicine. Identification of extreme resistance affords another opportunity. Here one could conceivably forego chemoradiation but more importantly, discover novel therapeutic targets to overcome resistance. In this vein we have

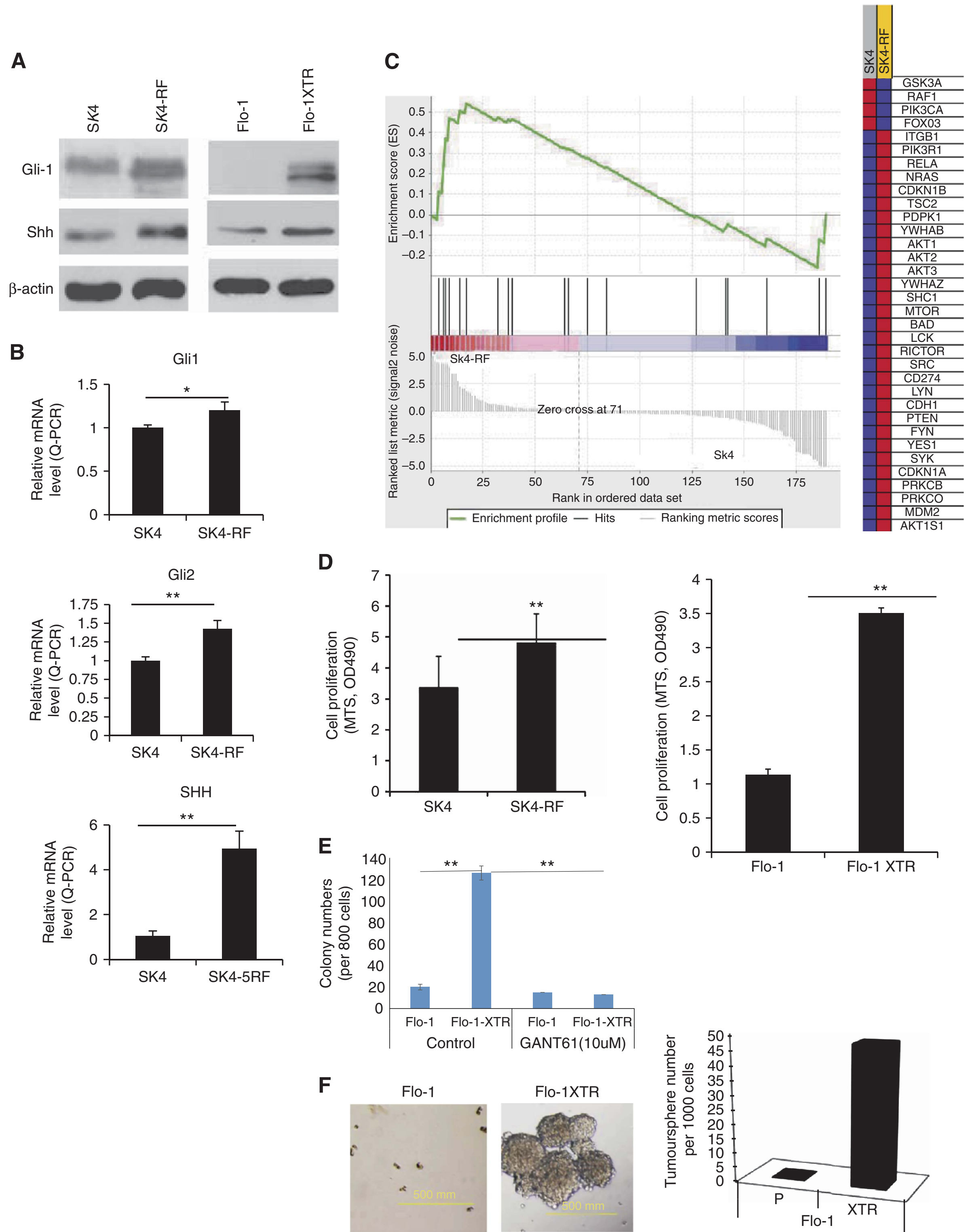

Figure 3. Expression of Hh signalling components is associated with chemoradiation resistance in OC cells. (**A**) Protein levels of Gli-1 and Shh were determined by immunoblotting in SK-4, Flo-1 EAC cells and their resistant cells SK4-RF and Flo-1-XTR; (**B**) mRNA level of Hh components-Gli-1, Gli-2 and Shh was determined by quantitative real-time PCR. (**C**) Gene set enriched analysis of RPPA proteomic data on Sk4 cells and their resistant cells SK4-RF on cell survival signalling and many genes control oncogenic signalling are enriched in chemoresistant OC cells (SK4-RF). (**D**) Cell proliferation of SK4 cells and their resistant cells SK4-RF and Flo-1 and its radiation resistant cells Flo-1XTR was measured using MTS assay. (**E**) Colony numbers of Flo-1 and Flo-1XTR cells treated with GANT61 or 100% ethanol as control, $^{**}P<0.01$. (**F**) Representative images of tumour spheres (left) and quantification of tumour sphere number (right) are shown in Flo-1 and Flo-1XTR cells. $^{*}P<0.05$, $^{**}P<0.01$.

recently discovered that anti-apoptotic agent ABT263 can overcome radiation/chemo resistance by targeting not only BCL-2 protein but also CSCs (Chen *et al*, 2015).

On the basis of our discoveries (Sims-Mourtada *et al*, 2006, 2007; Chen *et al*, 2007) and those of others regarding Gli-1's association with resistance (Zahreddine *et al*, 2014), we have

recently initiated a trial with Hh inhibitor with standard preoperative chemoradiation in patients whose OC's have ⩾5% LI of nuclear Gli-1 (NCT02530437). In addition, we acknowledge the complexity of cancer biology conferred by ability to reprogram and cross-talk with other pathways to emerge with aggressive phenotype. We believe, Gli-1 alone will not be able to define such complexity in each patient's tumour. Therefore, more work is needed to develop signature or signatures that might perform consistently when clinically implemented.

In conclusion, nuclear Gli-1 LIs correlated well with pathCR in TDS and TVS. We acknowledge that considerably more research would be needed before clinical implementation.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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