



Influence and mechanism of lung cavitation development on antiangiogenic therapy

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Background: Antiangiogenic agent-treated patients usually develop cavitation in their lung lesions. The clinical significance of lung cavitation development during antiangiogenic therapy has not been determined yet. Herein, we evaluated the clinical outcomes of patients who developed tumor cavitation following apatinib treatment and explored the mechanisms.

Methods: In this study (Clinical Trial No. NCT03629691), 187 patients (77 lung cancer and 110 gastric adenocarcinoma patients) who had progressed or relapsed after undergoing at least two lines of systemic therapy in accordance with the NCCN guidelines for primary or metastatic lung tumors were treated with apatinib at a dosage of 250 mg per day between February 1, 2015 and May 19, 2017. The effect of lung cavitation development on locoregional control (LRC), progression-free survival (PFS), and overall survival (OS) was analyzed with Kaplan-Meier estimates and compared with the log-rank test. Zebrafish experiments were used to study the anticancer mechanism of apatinib in different tumors. Western-blotting was used to analyze the expression of Cyclin D1, p53, HIF- α , and VEGFR before and after apatinib treatment in both normoxia and hypoxia.

Results: Cavitation development was beneficial in patients receiving apatinib therapy regardless of whether they had primary or metastatic lung cancer. Zebrafish experiments showed that apatinib inhibited tumor growth by both suppressing vascular growth and inhibiting cell proliferation. Vascular proliferation induced by the H1299 cell lines showed higher sensitivity to apatinib than that induced by the SCG-7901 cell line. However, apatinib showed weak tumor type selectivity on cell proliferation inhibition *in vivo*. Under hypoxic conditions, apatinib could not inhibit the protein expression of VEGFR and HIF- α in both cell lines; however, apatinib decreased the expression of cyclin D1 and P53 significantly.

Conclusions: Lung cavitation development is common with apatinib therapy and is a potential prognostic marker. Apatinib inhibits tumor growth by both vessel growth inhibition and proliferation inhibition.

Keywords: Apatinib; lung cavitation development; progression-free survival (PFS); hypoxia; anticancer mechanism

Submitted Jan 19, 2019. Accepted for publication Jun 26, 2019.

doi: 10.21037/tlcr.2019.07.01

View this article at: <http://dx.doi.org/10.21037/tlcr.2019.07.01>

Introduction

Lung cavitation often occurs in lung cancer patients who have failed multiple lines of chemotherapy and have been treated with antiangiogenic agents (1,2). It is often considered a risk factor for the deterioration of a patient's survival status (3). However, results may vary depending on the drug used. In a previous phase II trial, which included 67 non-small cell lung cancer (NSCLC) patients treated with bevacizumab, lung cavitation was associated with patient death (3). In another study of cediranib, Crabb *et al.* reported no clear link between clinical response and tumor cavitation in NSCLC patients (4). It is essential to study the relationship between lung cavitation development and antiangiogenic therapy to avoid serious adverse events and guide clinical treatments. Apatinib, a novel small-molecule vascular endothelial growth factor receptor (VEGFR) inhibitor, has been approved to treat advanced or metastatic chemorefractory gastric cancer in China (5,6). Recently, apatinib has been shown to have positive effects on multiple tumors, including lung cancer (6,7). According to our observations, apatinib-treated patients with lung lesions usually develop lung cavitation although the clinical significance of this finding has not been studied yet. Herein, we retrospectively reviewed apatinib-treated patients to evaluate the clinical significance of lung cavitation development during apatinib therapy (Clinical Trial Register No. NCT03629691). This is the first study concerning lung lesions of both primary and metastatic lung cancer. The study design was accepted as a poster at the 18th World Conference on Lung Cancer (WCLC, October 15–18, 2017, Yokohama, Japan P3.03-014).

Methods

Clinical study

Patients

Between February 1, 2015, and May 19, 2018, 433 adult patients with gastric adenocarcinoma, lung adenocarcinoma, and lung squamous cell carcinoma, who had failed multiple lines of chemotherapy and lacked a standard treatment regimen, received 250 mg oral apatinib daily at the Affiliated Hospital of Qingdao University. All patients had an Eastern Cooperative Oncology Group performance status of 0 to 1, with stable hepatic, hematological, and renal functions. Patients with cardiac disease or hemorrhagic disease were excluded. Other patient demographic and clinical data, including histopathology, age, gender, disease

stage, apatinib-administration inclusion and withdrawal dates, adverse events, oncologic clinical response, comorbid conditions, and smoking history, were retrieved from the Current Research Information System (CRIS) database. The study was approved by the institutional review board of the Affiliated Hospital of Qingdao University (No. QYFYEC 2015-006-07) and was registered as a clinical trial (No. NCT03629691).

Evaluation of clinical responses

All patients underwent a pretherapy chest computed tomography (CT) examination before apatinib treatment, which was performed an average of 7.3 days before initiating therapy, followed by chest CT examination at least once every four weeks. The primary endpoint was progression-free survival (PFS), and the secondary endpoints were the duration of locoregional control (LRC), overall survival (OS), quality of life, and safety. For metastatic lung cancer, LRC indicates local control of the metastatic lung tumors. Evaluation of the oncologic clinical responses was based on the alternate modified method published by Crabb *et al.* (4). This method was used to target lesions in which the longest diameter of any cavitation (zero if no cavity was present) was subtracted from the longest total lesion diameter, with each measurement taken in the same plane, to provide an alternate method to calculate the sum of the measurements for all target lesions.

Statistical methodology

The association between cavitation formation and the apatinib treatment effect was investigated. Number of patients, number of events, median time-to-event endpoints (LRC, OS, and PFS) and corresponding 95% confidence intervals (CIs) were evaluated for each subgroup; 95% CIs were calculated per Brookmeyer and Crowley method, and hazard ratios (HRs, including 95% CIs) were calculated using the Cox proportional hazards model. The Cox proportional hazards model was fitted to the data from all cavitation patients, with cavitation and biomarker statuses, as well as their interactions, as explanatory variables. The interactions between the treatment effect and biomarkers were tested using a two-sided Wald's test.

Zebrafish experiments

Zebrafish care and handling

Apatinib (purity >98%) was purchased from MERYER (Shanghai, China) and dissolved in dimethyl sulfoxide

(DMSO) to obtain stock concentrations of 5 mM and 500 μ M. Fetal bovine serum (FBS), phosphate-buffered saline (PBS), Roswell Park Memorial Institute basal medium 1640 (RPMI 1640 medium), penicillin, and streptomycin were purchased from Basal Media Technologies (Shanghai, China). H1299 and SCG-7901 cell lines were obtained from the American Type Culture Collection (ATCC) and fluorescently labeled with CM-DiI (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Cell viability was assessed by trypan blue staining before injection. The time length is indicated as days post-apatinib treatment (dpt). Transgenic zebrafish Tg (fli-1: EGFP) expressing enhanced green fluorescent protein (EGFP) in the endothelial cells were obtained from the Model Animal Research Center of Nanjing University. All zebrafish assays in our study were conducted before 120 hours post fertilization (hpf), as events before this time point are considered to be non-protected life stages in the European Union and are therefore accepted as an alternative to animal testing (8). Embryos and larvae were staged as described by Kimmel *et al.* (9). The labeled cells were injected into the yolk sac of the zebrafish embryo using stereoscopic microinjection (SMZ 745 T; Nikon, Japan).

Angiogenesis inhibition and cell proliferation suppression

Xenografted embryos were treated with 0.1, 0.5, and 0.25 μ M apatinib by soaking and incubating at 32 °C to observe angiogenesis inhibition and cell proliferation suppression. We monitored angiogenesis and tumor cell growth *in vivo* at 1, 2, and 3 dpt using an inverted fluorescence microscope (IX71; Olympus, Japan). Angiogenesis was observed using a confocal microscope (LSM710; ZEISS, Germany). The studies were approved by the Institutional Animal Care and Use Committee (IACUC) at the Affiliated Hospital of Qingdao University.

Statistical methodology

All statistical results were expressed as the mean \pm SEM and were generated using GraphPad Prism 5.0. The decreases/increases in the fold of change were analyzed using one-way ANOVA, followed by Dunnett's multiple comparison test. Significance was considered when P value was less than 0.005. *** indicates statistical significance $P < 0.005$, ** indicates $P < 0.01$, and * indicates $P < 0.05$. All experiments were performed in triplicate, and the independent

experiment was repeated at least three times.

Anticancer mechanism analysis

Cell culture

H1299 and SCG-7901 cell lines were routinely cultured in 175-mm flasks in serum-containing DMEM. Cells were placed in a modular incubator chamber (Billups Rothenberg, Inc., Del Mar, CA, USA) and flushed for 10 minutes with a gas mixture of 5% CO₂-95% N₂ to achieve and control hypoxic conditions; the final medium pO₂ value was consistently below the 0.5% to 1% range. The chamber was then sealed and placed at 37 °C in a conventional cell incubator.

Western-blot analysis

Protein expression in cultured cells was analyzed by western blotting, which was performed with equal amounts of whole-cell extracts obtained with RIPA buffer (Beyotime, China). Cells were washed with ice-cold phosphate-buffered saline (PBS) and collected by scraping. Protein extraction from cells was carried out. Protein levels in the extracts were quantified using the BCA protein assay (Beyotime, China). Western blots were generated and probed with specific primary antibodies against VEGFR-2, HIF- α , Cyclin D1, and P53. All antibodies were obtained from Abcam (SA). Blots were developed using the enhanced chemiluminescence detection system.

Results

Effect of lung cavitation development on survival

In total, 187 patients with pulmonary tumor lesions were included in our study. Seventy-seven patients had lung cancer, and 110 had gastric adenocarcinoma (*Figure 1*). *Table 1* shows the baseline characteristics of primary and metastatic lung cancer patients. The median age of the study subjects was 58.2 years (range, 33–79 years). Age ($P=0.35$), sex ($P=0.23$), tumor stage ($P=0.06$), pneumonia status ($P=0.59$) and adverse event occurrences ($P=0.76$, 0.48, and 0.14 for hypertension, skin rash, and proteinuria, respectively) did not differ between the primary and metastatic lung cancer cohorts. More primary lung cancer patients than metastatic lung cancer patients underwent pulmonary surgery ($P=0.002$) or radiotherapy ($P<0.001$). *Figure 2* shows cases of lung cavitation development during apatinib therapy. Survival analysis indicates that lung

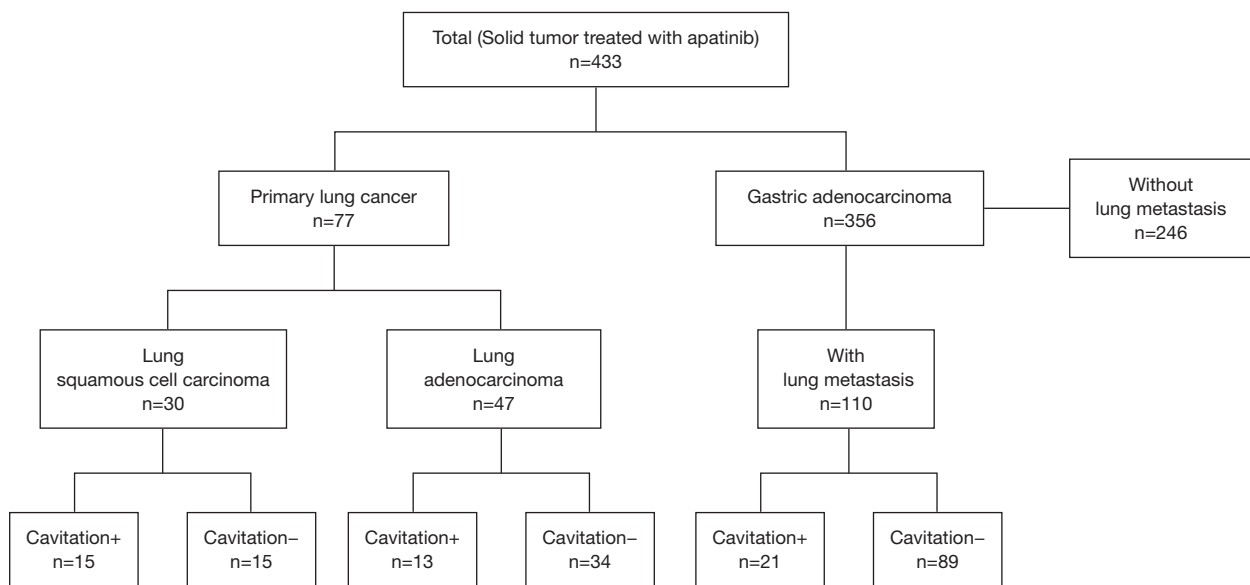


Figure 1 Trial profile.

Table 1 Characteristics of patients with lung cancer

Characteristics	Total patients with pulmonary tumor lesions (n=187)					
	Primary lung cancer				Gastric cancer with lung metastasis	
	Total	Adenocarcinoma	SCC	P	Total	P
Age (>60 years)	31	21	10	0.54	21	0.35
Sex (female)	34	24	10	0.26	23	0.23
Tumor stage (III)	4	2	2	0.56	15	0.06
Pulmonary surgery	38	21	17	0.13	30	0.002*
Radiotherapy	71	45	26	0.87	29	<0.001*
Pneumonia	15	9	6	0.74	25	0.59
Adverse events						
Hypertension	67	44	23	0.34	94	0.76
Skin rash	71	46	25	0.47	98	0.48
Proteinuria	49	30	19	0.56	58	0.14

*, P<0.05. SCC, lung squamous cell carcinoma.

cavitation development improved LRC, PFS, and OS, regardless of whether patients had primary or metastatic lung cancer (Figures 3,4). According to Table 2, in patients without lung cavitation, gastric cancer patients benefited more from apatinib therapy than did primary lung cancer patients. However, when lung cavitation developed, the selectivity of apatinib became weak. In the primary lung

cancer cohort, histology did not influence the effect of apatinib therapy (Table 3).

Anticancer mechanism of apatinib

Angiogenesis inhibition

Figure 5A shows vessel growth inhibition properties of

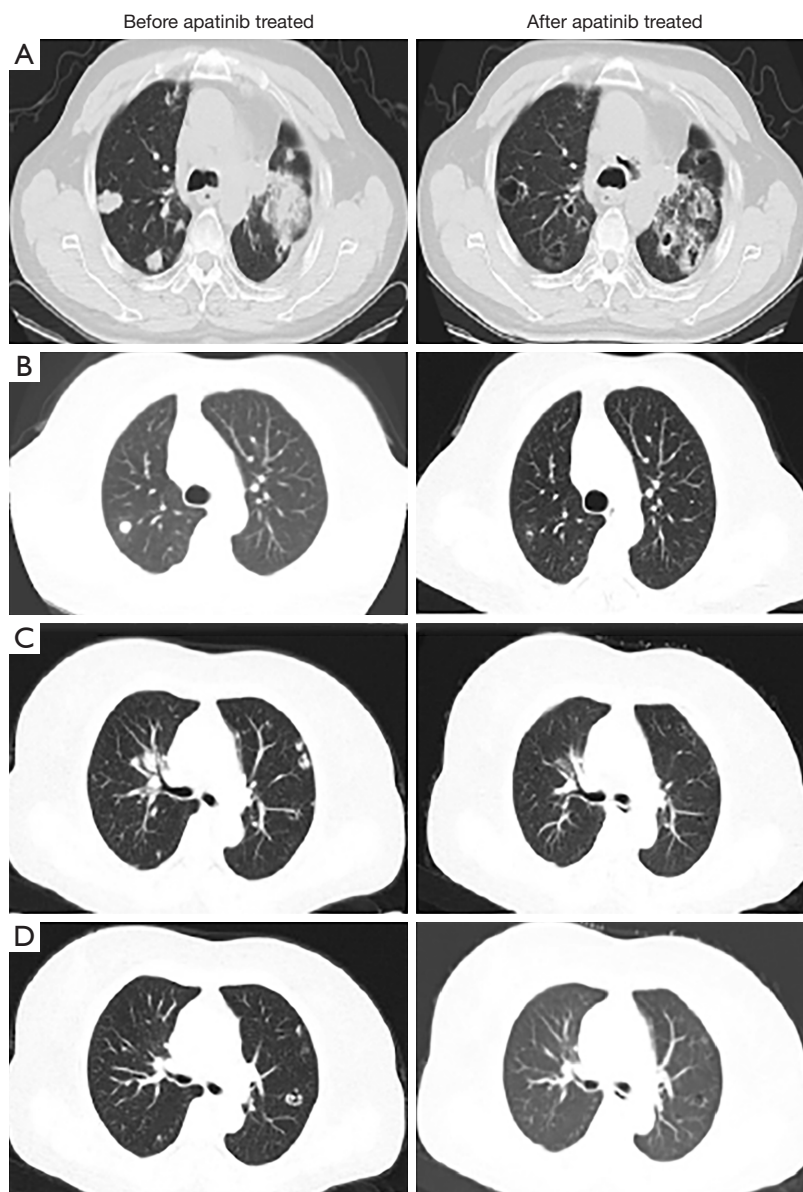


Figure 2 Chest CT scans of patients. (A) A 60-year-old man with stage IV lung squamous cell carcinoma treated with apatinib. His baseline chest CT scan prior to therapy demonstrated a solid dominant mass in the left lung, and four nodules were noted in the right lower lobe. A follow-up CT scan at 1.5 months after apatinib therapy demonstrated that a cavity developed in the left lung within the dominant mass, decreasing in the size of the mass. The four nodules in the right lung developed cavitations. (B) A 60-year-old woman with stage IV lung adenocarcinoma who underwent right lower lobectomy 4 years ago, presenting with histologically confirmed recurrent disease in the right lung nodules. Her baseline chest CT scan prior to therapy demonstrated pleural nodularity along the right lung and small faint nodules in the left lower lobe. A follow-up CT scan at 5 months after apatinib therapy indicated the formation of a cavitation in the right lung. (C) A 52-year-old man with stage IV gastric cancer with lung metastasis who presented with histologically confirmed recurrent disease in the lung nodules. His baseline contrast chest CT scan prior to therapy demonstrated pleural nodularity along the left lung. Follow-up CT scan at 3 months after apatinib therapy indicated cavitation in these nodules. (D) A 55-year-old woman with stage IV gastric cancer with lung metastasis who presented with histologically confirmed recurrent disease in lung nodules. Her baseline contrast-enhanced chest CT scan prior to therapy demonstrated pleural nodularity along the right lung and small faint nodules in the left lower lobe. Follow-up CT scan at 7 months after apatinib therapy indicated cavitation in the nodule in the right lung.

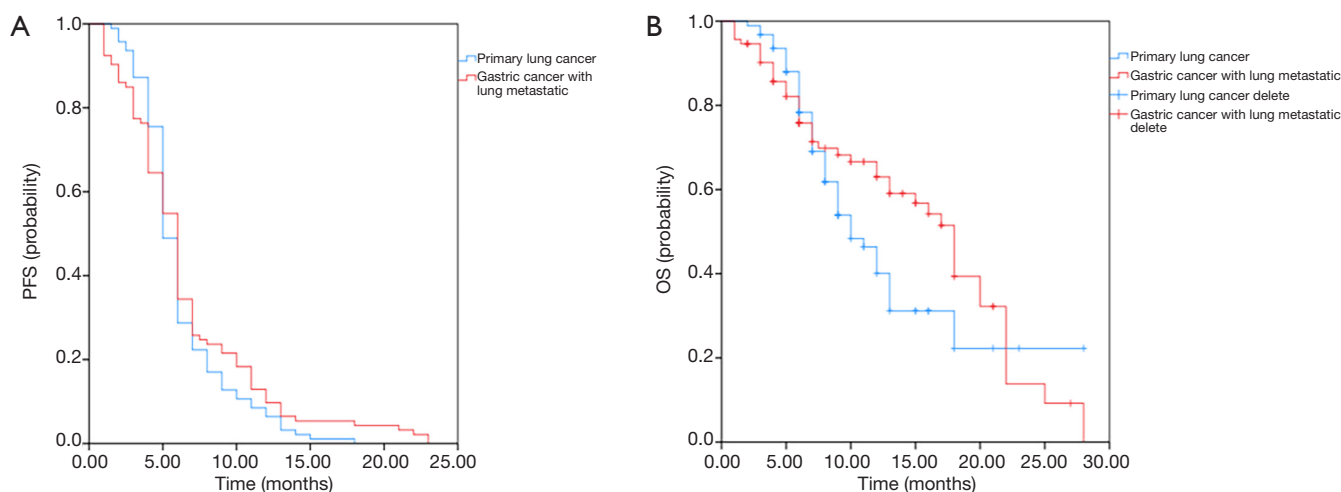


Figure 3 Survival analysis of apatinib treated primary lung cancer and gastric cancer patients. (A) Survival analysis of apatinib-treated primary lung cancer patients; (B) survival analysis of apatinib-treated primary gastric cancer patients with lung metastasis.

apatinib in different tumor types. As expected, apatinib inhibited vessel growth induced by both cell lines early at 1 dpt. When the dose was increased from 0.25 to 1 μM , apatinib decreased the development of H1299-cell-induced intact subintestinal vessels (SIVs) in zebrafish from a basket to several branches. However, for SCG-7901-cell-induced SIVs, the changes were not significant. Vessel length of control zebrafish was set as the baseline and was normalized to 1. At 1 dpt the H1299-cell-induced SIV lengths were reduced to 0.82, 0.68, and 0.66 fold in the 0.25, 0.5, and 1 μM apatinib treatment groups respectively (*Figure 5B*). In contrast, SCG-7901-cell-induced SIV lengths reduced to 0.84, 0.76, and 0.69 fold in the 0.25, 0.5, and 1 μM apatinib treatment groups respectively (*Figure 5C*). Taken together, these results indicate that the vascular proliferation induced by H1299 cell lines showed higher sensitivity to apatinib than that caused by the SCG-7901 cell lines.

Cell proliferation suppression

H1299 and SCG-7901 cells were xenografted into zebrafish soaked in apatinib 0.25–1 μM at 1–3 dpt. The cell number at 0 dpt was set as the baseline and normalized to 1. At 3 dpt, the H1299 cells proliferated by 1.93 fold in the control group, and 1.85, 1.56 and 1.50 fold in the 0.25, 0.5, and 1 μM apatinib treatment groups, respectively (*Figure 6*). SCG-7901 cells exhibited a 1.61-fold increase in the control group at 3 dpt, but were 1.49, 1.27, and 1.25 fold in the 0.25, 0.5 and, 1 μM apatinib treatment groups, respectively (*Figure 7*). These results indicate the weak selectivity of

apatinib in cell proliferation inhibition *in vivo*.

Molecular mechanism analysis

Protein expression of VEGFR, HIF- α , Cyclin D1, and P53 in control and 0.5 μM apatinib treatment groups of H1299 and SCG-7901 cell lines in normoxic and hypoxic environments was evaluated. The results are described in *Figure 8*. In normoxia, apatinib decreased the protein expression of VEGFR and P53 but did not affect the expression of Cyclin D1 or HIF- α in both cell lines. In hypoxia, the protein expression of Cyclin D1 and P53 in both cell lines treated with apatinib was significantly lower than the control. However, the protein expression of VEGFR was not decreased. Furthermore, the expression of HIF- α was increased.

Discussion

Lung cavitation development is a potential prognostic biomarker of apatinib therapy

Apatinib is the first small-molecule VEGFR inhibitor approved to treat advanced or metastatic chemorefractory gastric cancer in China (10). Additionally, it has been confirmed to increase the duration of LRC and survival in patients with multiple solid tumors (5-7,11-21). As we have observed, many patients treated with apatinib develop cavitation in their lung lesions. Previously, several clinical trials evaluated the effect of the cavitation status on the efficacy of antiangiogenic drug treatment in patients with

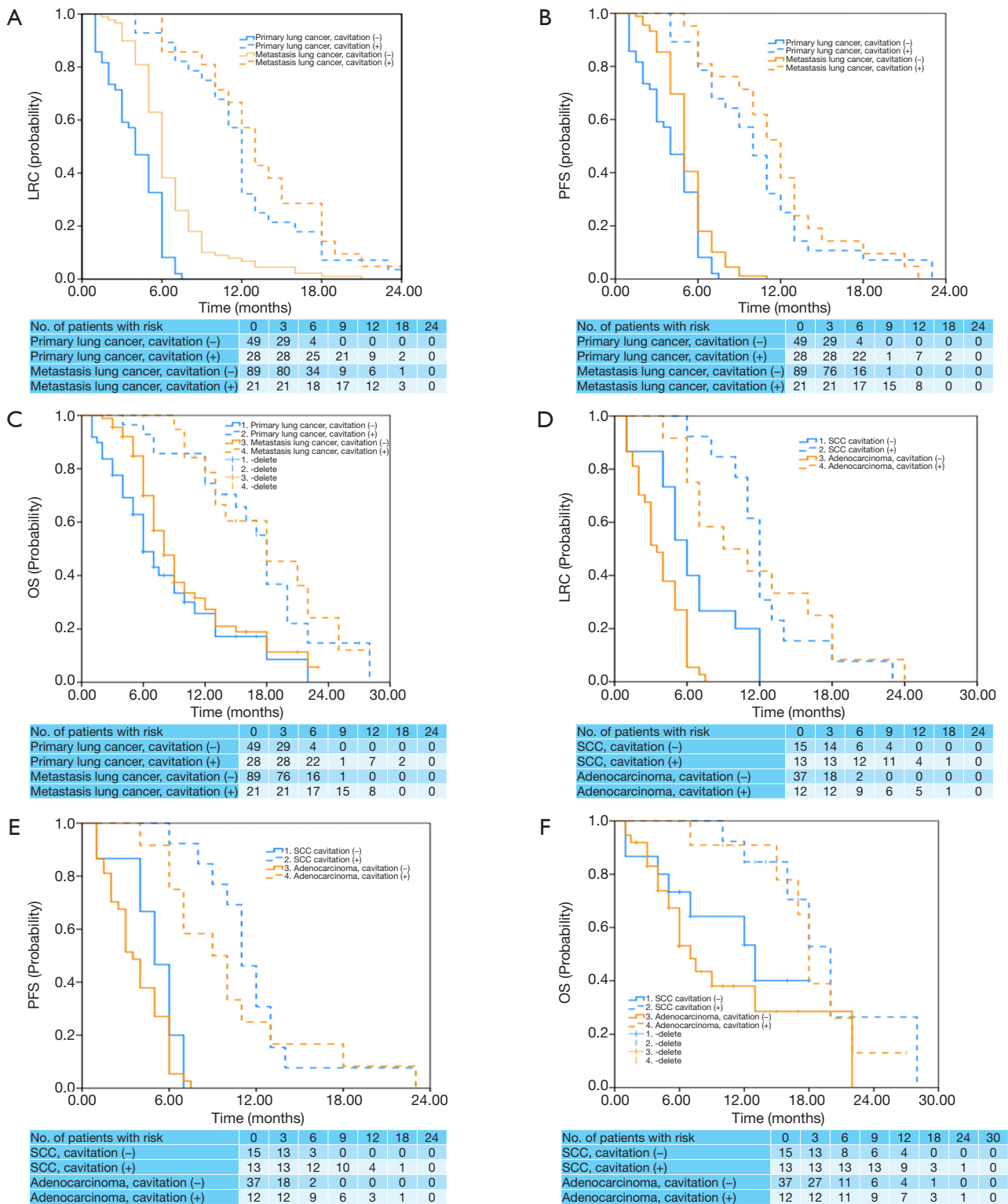


Figure 4 Kaplan-Meier plots of LRC, PFS, and OS (A,B,C) in primary and metastatic lung cancer patients with or without lung cavitation, and (D,E,F) in primary lung cancer patients with or without lung cavitation. Because there was minor overlap among the Kaplan-Meier curves, which may have resulted from the small sample size, additional statistical analyses were conducted to ensure there was no strong deviation from the original proportional hazards assumption. Both log-log survival plots and time-dependent Cox models confirmed this assumption was not violated. LRC, locoregional control; PFS, progression-free survival; OS, overall survival

Table 2 Efficacy outcomes of apatinib-treated patients with lung cancer grouped by their different primary tumor types primaries and cavitation statuses

Outcome	6-month rate (%)						HR (95% CI)			
	Primary lung cancer		Metastasis lung cancer		Cavitation effect [#]		Cavitation effect [#]		Tumor effect [†]	
	Cavitation-	Cavitation+	Cavitation-	Cavitation+	Primary	Metastasis	Cavitation-	Cavitation+	Cavitation-	Cavitation+
LRC	8.2	89.3	38.2	85.7	0.063** (0.024 to 0.168), P<0.001	0.309** (0.185 to 0.517), P<0.001	0.785 (0.444 to 1.387), P=0.404	0.409** (0.281 to 0.596), P<0.001		
PFS	8.2	78.6	18	81	0.125** (0.059 to 0.265), P<0.001	0.129** (0.061 to 0.220), P<0.001	1.061 (0.767 to 1.469), P=0.720	1.259* (1.048 to 1.512), P=0.014		
OS	48.9	92.9	70	94.7	0.293** (0.158 to 0.543), P<0.001	0.316** (0.163 to 0.612), P=0.001	1.00 (0.482 to 2.076), P=1	0.699 (0.459 to 1.066), P=0.096		

*, P<0.05; **, P<0.01; [#], evaluation of the effect of treatment in different cavitation status. HR <1 favored cavitation formed; [†], evaluation of the effect of treatment in different tumor types. HR <1 favored metastatic lung cancer. HR, hazard ratio; LRC, locoregional control in lung herein; OS, overall survival; PFS, progression-free survival.

Table 3 Efficacy outcomes of apatinib-treated patients with primary lung cancer grouped by their different histologies and cavitation statuses

Outcome	6-month PFS rate (%)				HR (95% CI)			
	SCC		Adenocarcinoma		Cavitation effect [#]		Histology effect [†]	
	Cavitation-	Cavitation+	Cavitation-	Cavitation+	SCC	Adenocarcinoma	Cavitation-	Cavitation+
LRC	40	92.3	5.4	75	0.085** (0.021 to 0.338), P<0.001	0.020* (0.001 to 0.383), P=0.009	0.970 (0.431 to 2.179), P=0.940	0.436 (0.171 to 1.114), P=0.083
PFS	20	92.3	5.4	75	0.165* (0.054 to 0.505), P=0.002	0.020* (0.001 to 0.383), P=0.009	0.970 (0.431 to 2.179), P=0.940	0.573 (0.240 to 1.369), P=0.210
OS	73.3	92.3	53.2	100	0.111* (0.028 to 0.443), P=0.002	0.358 (0.125 to 1.024), P=0.055	0.635 (0.238 to 1.696), P=0.940	0.987 (0.298 to 3.263), P=0.983

*, P<0.05; **, P<0.01; [#], evaluation of the effect of treatment in different cavitation status. HR <1 favored cavitation formed; [†], evaluation of the effect of treatment in different tumor types. HR <1 favored adenocarcinoma histology. PFS, progression-free survival; HR, hazard ratio; LRC, locoregional control in lung herein; OS, overall survival.

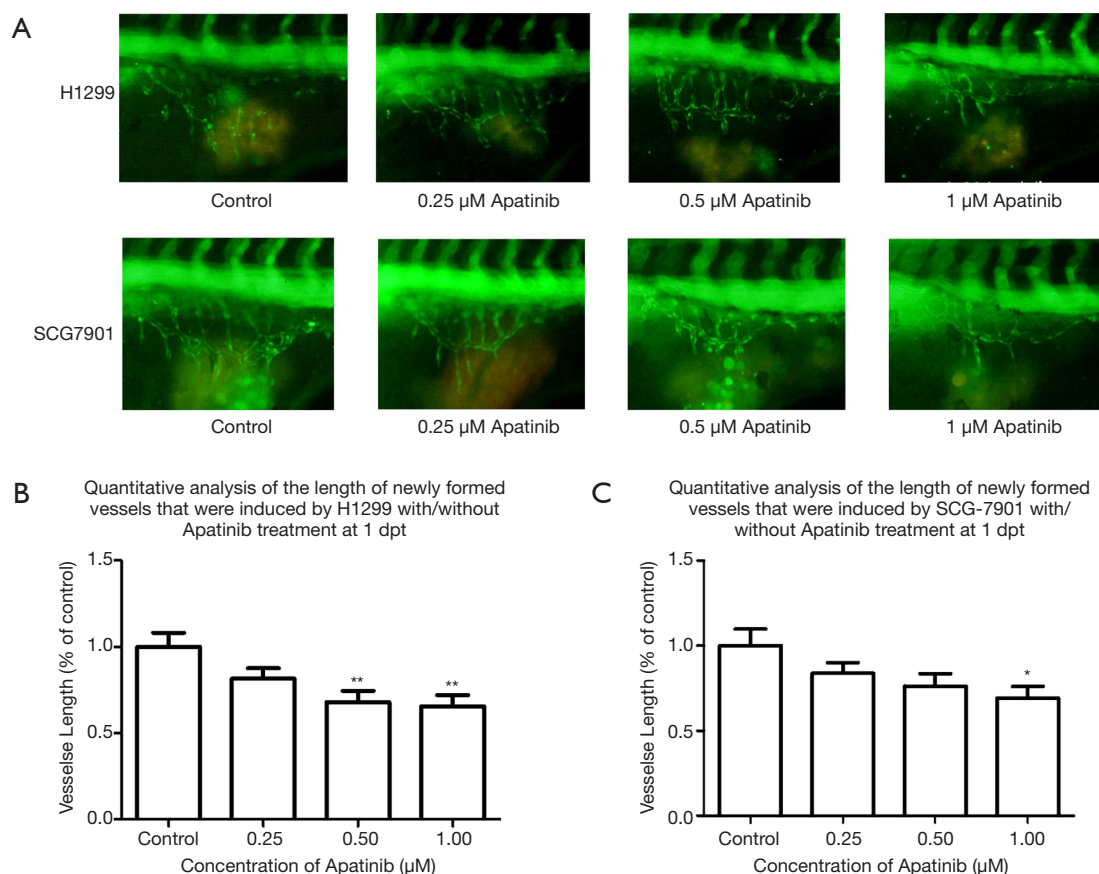


Figure 5 Angiogenesis inhibition analysis. (A) The angiogenesis inhibition with 0–1 μM apatinib in different tumor types. Apatinib could suppress vessel growth induced by the H1299 and SCG7901 cell lines in a dose dependent manner at 1 dpt (days post apatinib treated). (B) Quantitative analysis of the lengths of newly formed vessels induced by H1299 cell lines without/with apatinib treatment. (C) Quantitative analysis of the lengths of newly formed vessels induced by SCG-7901 cell lines without/with apatinib treatment. * $P < 0.05$, ** $P < 0.01$.

NSCLC (1-4). However, the relationship between tumor cavitation development and clinical response remains uncertain, while studies exploring the potential lung cavitation development induced by apatinib treatment are lacking. To explore the clinical significance of this phenomenon during apatinib therapy, we evaluated the predictive roles of lung cavitation development during apatinib treatment in both primary and metastatic lung cancers. This is the first study of lung cavitation development in metastatic tumors. According to our results, regardless of whether patients had primary or metastatic lung cancer, lung cavitation development was associated with better efficacy of apatinib, and may be a potential prognostic biomarker in apatinib therapy.

Apatinib inhibited tumor growth by both vascular proliferation suppression and cell proliferation inhibition

Among patients without lung cavitation development, primary lung cancer patients benefited more from apatinib therapy than metastatic lung cancer patients. However, in patients with lung cavitation, tumor types did not influence the clinical response to apatinib treatment. In the vascular proliferation suppression experiments with zebrafish, SIVs induced by lung tumor cells were more sensitive to apatinib than those caused by gastric tumor cells, which followed the clinical outcomes of the patients without lung cavitation development. Also, cell proliferation inhibition *in vivo* showed weak tumor type selectivity for apatinib, which

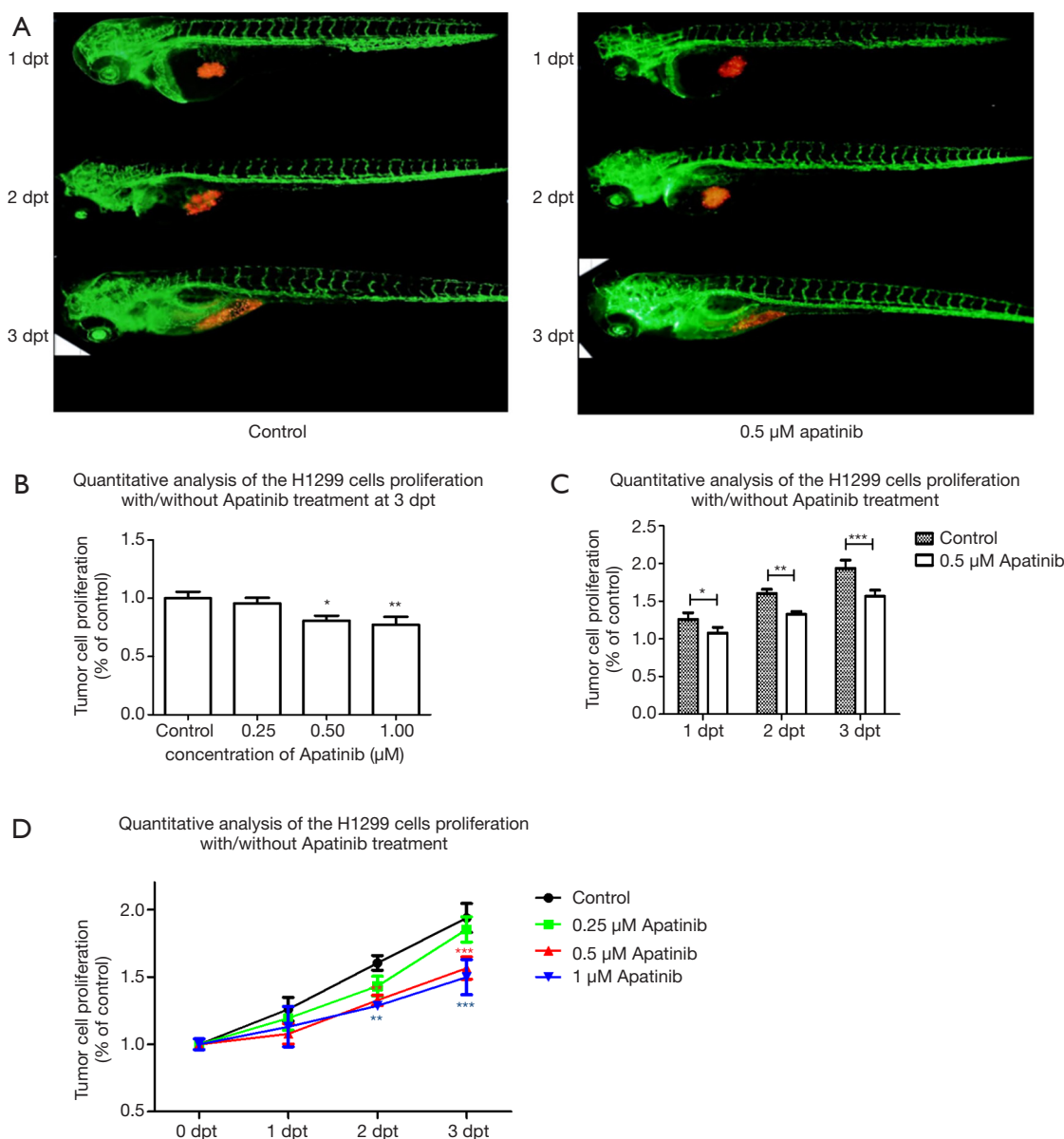


Figure 6 Suppression H1299 cell proliferation by apatinib. (A) The cell proliferation suppression mediated by 0.5 μM apatinib at 1–3 dpt (days post apatinib treated). Apatinib could suppress cell proliferation of H1299 cells at a concentration of 0.5 μM. (B) Suppression of H1299 cell proliferation by apatinib in a dose dependent manner at 3 dpt. Treatment with 1 μM apatinib significantly inhibited the proliferation of H1299 cells. (C) Suppression of H1299 cells proliferation with 0.5 μM apatinib in a time dependent manner. At 2 dpt, apatinib significantly inhibited H1299 cell proliferation. (D) Cell growth curves of H1299 cells without or with apatinib treatment at 0–3 dpt. *P<0.05, **P<0.01, ***P<0.005.

was consistent with the clinical results of the patients with lung cavitation development. As the zebrafish experiments showed, apatinib inhibited tumor growth by both vascular proliferation suppression and cell proliferation inhibition. Interestingly, in a hypoxic environment, apatinib increased

the expression of HIF-1α and did not affect VEGFR expression, which was quite different from the effects observed in a normoxic environment. According to these results, we deem it necessary to further study the anticancer mechanism of apatinib.

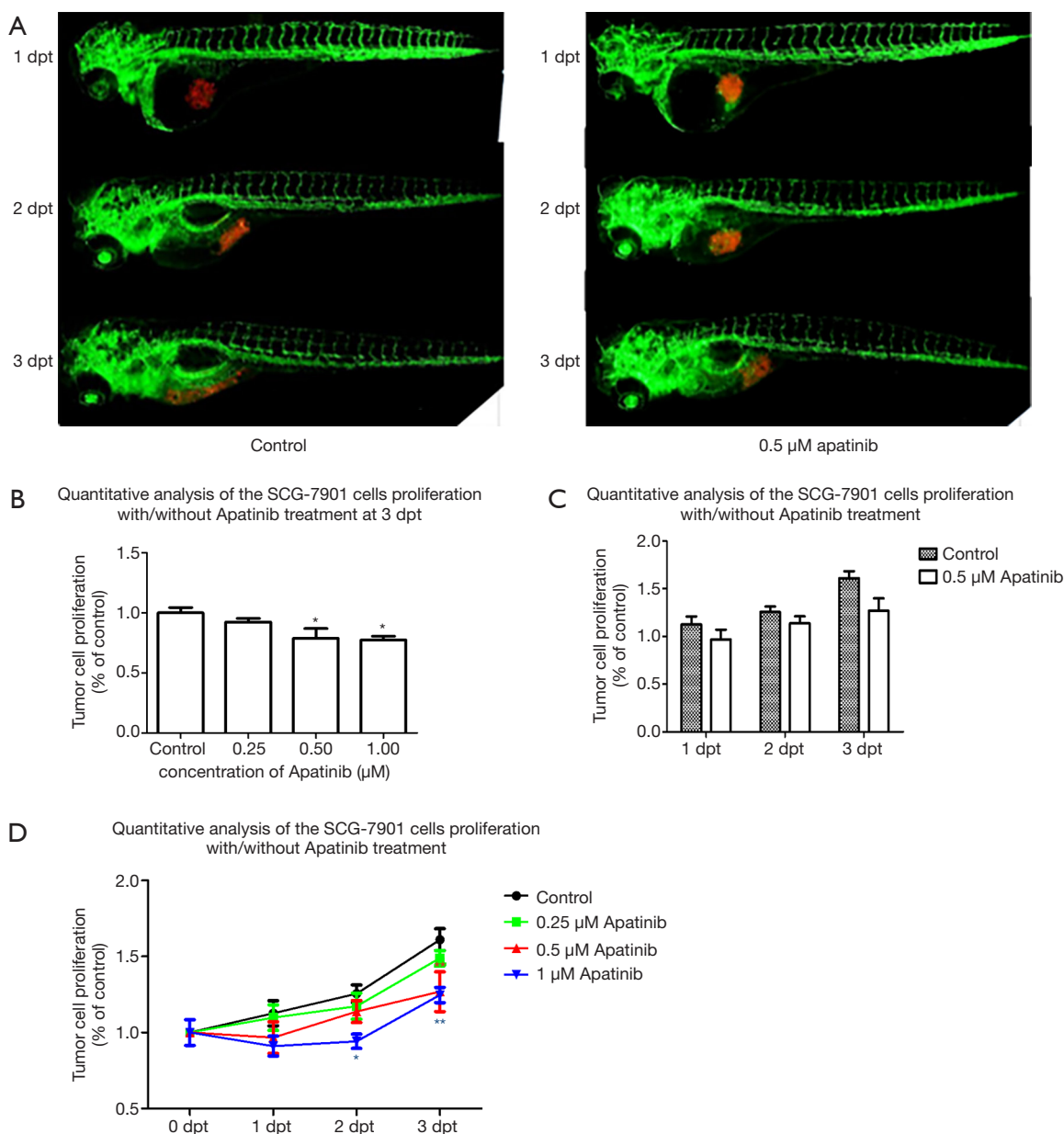


Figure 7 Suppression of SCG-7901 cell proliferation by apatinib. (A) The cell proliferation suppression mediated by 0.5 μM apatinib at 1–3 dpt (days post apatinib treated). (B) Suppression of SCG-7901 cell proliferation by apatinib in a dose dependent manner at 3 dpt. (C) Suppression of SCG-7901 cells proliferation with 0.5 μM apatinib at 1–3 dpt. (D) Cell growth curves of SCG-7901 cells without or with apatinib treatment at 0–3 dpt. * $P < 0.05$, ** $P < 0.01$.

Limitation

This study had several limitations. This was a retrospective analysis of the cavitation status in a previously unselected population, and the patient numbers in some groups were small. Therefore, some differences in tumor baseline characteristics could not be controlled. *In vivo* experiments

were performed only in zebrafish; more cellular trials and mouse studies should be designed for further research. Because better outcomes are expected in patients with lung cavitation, it may take years before enough progression events or deaths occur to facilitate assessing efficacy in this population. When available, the final dataset, including

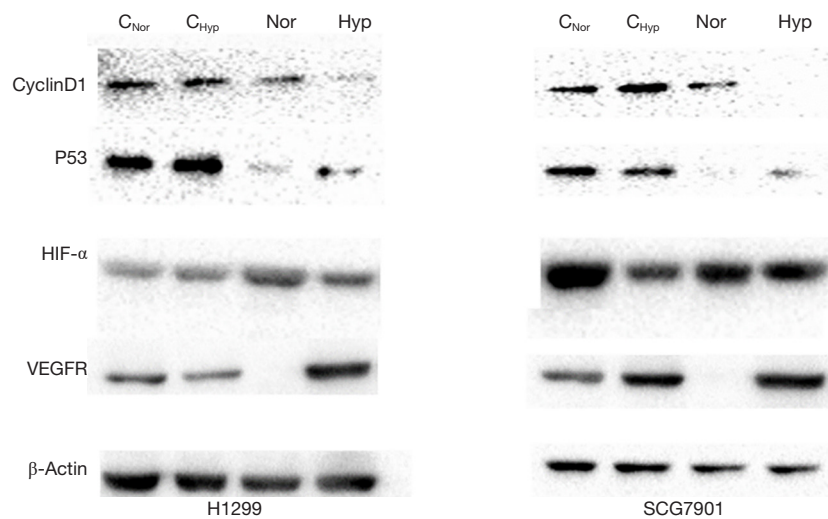


Figure 8 Protein expression of VEGFR, HIF- α , Cyclin D1 and P53 in control and 0.5 μ M apatinib treated H1299 and SCG7901 cells in a normoxic or hypoxic environments. C_{Nor}, control in normoxic environment; C_{Hyp}, control in hypoxic environment; Nor, 0.5 μ M apatinib treated cells cultured in normoxic environment; Hyp, 0.5 μ M apatinib treated cells cultured in hypoxic environment.

the effectiveness and late toxicity endpoints, will provide valuable insight into differences in survival and quality of life between patients who develop cavitation and those who do not.

Conclusions

In conclusion, the analysis of this trial showed that cavitation development was beneficial in apatinib-treated patients and functioned as a prognostic biomarker.

Acknowledgments

Funding: This work was supported by the Taishan Scholar Foundation (grant tshw 201502061 to X Zhang), Qingdao People's Livelihood Science and Technology Program (grant 16-6-2-3-nsh to X Zhang and 18-2-2-74-jch to M Jiang), Chinese Postdoctoral Science Foundation (2017M6122218 to M Jiang).

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are

appropriately investigated and resolved. The study was approved by the institutional review board of the Affiliated Hospital of Qingdao University (No. QYFYEC 2015-006-07) and written informed consent was obtained from all patients.

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Cite this article as: Jiang M, Zhang C, Liu D, Wang Y, Wang H, Li T, Hou H, Zhou N, Zhu J, Lv H, Zhang C, Fang B, Zhang X. Influence and mechanism of lung cavitation development on antiangiogenic therapy. *Transl Lung Cancer Res* 2019;8(4):500-512. doi: 10.21037/tlcr.2019.07.01