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探討血管生成相似素1在肺癌中參與腫瘤代謝的分子機制

The role of Angiopoietin-like 1 in lung cancer progression via altering metabolism

曾士桓

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本論文係曾士桓君（學號 R98447005）在國立臺灣大學毒理學研究所所完成之碩士學位論文，於民國 101 年 7 月 25 日承下列考試委員審查通過及口試及格，特此證明

口試委員：郭明良（指導教授簽名）

教授

教授

教授

系主任、所長　教授

誌謝

從未想過我竟然也會有碩士畢業的一天，心中的感動到現在都還不能平息，從大學畢業後，當兵、TOEFL、GRE、出國念書、回國、補習、念書、考研究所似乎都還只是昨天的事情，三年的時間，說長不長，說短不短，雖然比一般碩士班念得更久，但是其實我學得比一般碩士生多更多，這三年來，從一個連細胞都不會養的呆呆學生，變成了現在碩士班畢業，其中的轉變，真不是三言兩語可以形容。這段期間，要感謝的人很多，最感謝的是我的家人，這段路上有他們支持著我，才能讓我好好的完成學業。在做學問的過程中，最感謝郭老師的指導，讓我在邏輯思考上有著顯著的進步與增長了視野。在實驗上最感謝倉志的指導，在這些日子來，不管是是做實驗的技巧與方法，甚至是做人處事的方法與態度，都有更深一層的體會，讓我知道人越是在逆境中，就越要能自我成長與突破。別人常跟我說，我有一個好學長，嗯，我想這是真的。我常用Lou Gehrig在退休時的一段演說來代表我是如此的幸運的遇到倉志"Yet today I consider myself the luckiest man on the face of this earth." 常言到，"遇到好學長，帶你上天堂。" 我想我能畢業就跟上天堂沒什麼兩樣了吧。除了倉志外，還要感謝阿宅、阿蛋、詩婷、凱君、淑娟、家賦、孟真、小銘、鄴方、泡泡、小白、品均、品妤、嘉雯、敬一這些cancer stem cell lab的好夥伴，一起走過的這段時間，替一成不變的實驗室生活增添了無數的歡樂與驚喜。還要感謝國泰、啟寬、奕捷、余姵、翰博、尹琳、才甯、代謝體組的郭錦樺老師與曾宇鳳老師和他們的學生：冠元、威青、松政、依琳、翰駿、羽晨、國清、天爵在實驗上給與的支援與指導，讓我在完成這篇論文上更加的順利與完整。另外還有要感謝血管新生中心研8-29的瑞茹、宜宋、又新、惠宣、亭玉，那段一起吃喝玩樂的歲月，就好像大家昨天才聚在一起過，真實也從未走遠。

最後我用我最喜歡的科學家 Thomas Alva Edison 說過的話來勉勵自己與閱讀此篇論文的讀者：

There's a way to do it better - find it.

曾士桓 於 2012年 夏

中文摘要

癌症 (惡性腫瘤)一直以來都是國人十大死因之首，其中，得到肺癌的比例也都居高不下，因此肺癌的治療是相當重要的課題。根據的研究指出，癌細胞的代謝已經被認為是一個用做藥物設計治療癌症的新方向。在此篇研究中，我們企圖去呈現一個與血管新生相關的蛋白:血管相似素 1 可能能夠藉由改變癌細胞的代謝而抑制了肺癌的進程。首先，我們利用了代謝體學的方法分析了 CL1-5 肺癌細胞株在血管相似素 1 過量表現的與原本細胞株的差異，進而發現了過量表現血管相似素 1 的 CL1-5 肺癌細胞可能由麩醯胺酸酶 2 (Glutaminase 2)改變細胞內麩醯胺酸 (glutamine)的表現量。而且，我們更進一步的發現在抑制了麩醯胺酸酶 2 的 mRNA 表現後，會增加其原本血管相似素 1 所抑制的癌症進程如侵犯力 (invasion)、移動力 (migration)以及細胞的非貼附性生長 (anchorage independent growth)等。為了證明我們的觀察是否也會在活體內發生，我們利用了免疫不全 (SCID)的小鼠做了原位模式 (orthotropic model)的動物實驗進行應證，發現了與原本細胞實驗有類似的結果。進一步的，我們想探討血管相似素 1 可能會透過什麼樣的機制誘導了麩醯胺酸酶 2 的表現。因為血管相似素 1 是分泌型的蛋白，於是我們推測血管相似素 1 可能會透過一系列的訊息傳遞來達到誘導麩醯胺酸酶 2 的表現，然而在麩醯胺酸酶 2 的啟動子區(promoter region) 找到了轉錄因子 FOXOs 的同源辨認位 (cognate recognition site)，進而發現了血管相似素 1 是透過了磷酸化的 Erk 路徑來調控 FOXO 家族中的 FOXO3a 而影響麩醯胺酸酶 2 的表現。再者，為了證明血管相似素 1 與麩醯胺酸酶 2 在臨床上是否也具有相關性，也想探討這 2 個蛋白與存活率的關係，於是我們針對了 82 位肺癌病人的組織切片做免疫組織染色，經過統計後發現血管相似素 1 與麩醯胺酸酶 2 呈正相關且這 2 種蛋白的表現都和存活率有顯著的正相關性。以上的研究指出了血管相似素 1 可能透過改變了麩醯胺酸酶 2 的代謝角色造成了癌症的進程受到抑制。未來希望能夠透過此篇的研究結果，能發展出肺癌治療的新方法或新的藥物，增加肺癌病人的存活率以及治癒率。

關鍵詞: 1.肺癌

2.血管新生

3.血管相似素 1

4.麩醯胺酸

5.癌細胞代謝

Abstract

Cancer is the most deadly disease in Taiwan. Among all type of cancer, lung cancer is one of the most lethal types. Therefore, searching the cures of lung cancer is an important issue. According to recent research, cancer cells metabolism are considered as a new direction of drug design for cancer therapy. In this study, we present a new biological function of an anti-angiogenesis protein, Angiopoietin-like 1 (ANGPTL1), can influence lung cancer progression via affecting lung cancer cells metabolism. We use LC/MS to analyze the whole metabolites in overexpression ANGPTL1 and wild type cells. The profile indicates that intracellular glutamine is lower in overexpression ANGPTL1 cells. After screen some glutamine-related enzymes, we found that the level of intracellular glutamine changed through the glutamine catabolic enzyme Glutaminase 2 (GLS2). Then, we evaluated the function of ANGPTL1. The results showed that ANGPTL1 can inhibit the invasion, migration and anchorage independent abilities. Further, knockdown GLS2 will restore those abilities which are inhibited by ANGPTL1. In animal model, we found that ANGPTL1 can repress intra-pulmonary metastasis and nodules numbers but knockdown ANGPTL1-induced GLS2 can regain lung cancer intra-pulmonary metastasis *in vivo* and increase the nodules. We further investigated the possible mechanism of how ANGPTL1 can induce GLS2. The data showed that ANGPTL1-inhibited mobility and anchorage independent ability of lung cancer cells were found to required upregulation of GLS2 through inhibiting phospho-Erk/FOXO3a pathway. Clinically, we found that ANGPTL1 is positive correlation with GLS2 and their expression are inversely correlated with poor clinical outcomes. Above data suggested that ANGPTL1 has a new role in glutamine catabolism and can inhibit tumor progression by inducing GLS2. In conclusion, our findings provide a potential role of ANGPTL1 involved in regulation of tumor metabolism pathway that further to affect tumor progression in lung cancer.

Key word: 1. Lung cancer

2. Angiogenesis

3. Angiopoietin-like 1

4. Glutamine

5. Cancer cell metabolism

Content

Introduction

1-1 Lung cancer

Cancer has been the most deadly disease in Taiwan in last decades. Among all types of cancer, lung cancer is one of the most lethal types. There are 2 main types of lung cancer, non-small cell lung cancer (~85%) and small cell lung cancer (~15%). Non–small-cell lung cancer can be divided into three major histologic subtypes: squamous-cell carcinoma, adenocarcinoma, and large-cell lung cancer. Smoking causes all types of lung cancer but is most strongly linked with small-cell lung cancer and squamous-cell carcinoma; adenocarcinoma is the most common type in patients who have never smoked. Despite advances in early detection and standard treatment, non–small-cell lung cancer is often diagnosed at an advanced stage and has a poor prognosis (Herbst et al., 2008). Lung cancer risk also are increased in inherited cancer syndromes caused by rare germ-line mutations in p53 (Hwang et al., 2003), retinoblastoma (Sanders et al., 1989), and epidermal growth factor receptor (EGFR) gene (Bell et al., 2005). These genes instability caused cures of lung cancer are sometimes inefficient and recurance. The new cures of lung cancer are still been needed which can probably be reformed by a new comprehension of the molecular function and mechanism.

1-2 The characteristics of cancer and cancer metabolism

Generally speaking, the cancer cells are characterized as several unique biological functions during the multistep progression. The hallmarks constitute an organizing principle for rationalizing the complexities of neoplastic disease. They include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis,

and activating invasion and metastasis (Hanahan and Weinberg, 2011). These hallmarks of cancer are considered as the therapeutic targets and have been proven that the small molecular inhibitors of the hallmarks regulators are efficient to inhibit tumor growth, tumor invasion, tumor migration, and angiogenesis in vitro, in vivo, even in clinical trials (Atkins and Gershell, 2002; Hanahan and Weinberg, 2011). However, according to recent research, cancer cells are different from normal cells not only in these hallmarks but also the metabolism reprogramming (D'Alessandro and Zolla, 2012; Hsu and Sabatini, 2008; Locasale and Cantley, 2010; Vander Heiden, 2011; Vander Heiden et al., 2009). The metabolism reprogramming phenomena are even considered as the new direction of anti-cancer drug development (Hanahan and Weinberg, 2011; Pathania et al., 2009; Vander Heiden, 2011) because of the following reasons. First, metabolism is a direct response to growth factor signaling. For example, the reprogramming of cellular metabolism toward macromolecular synthesis is critical to supplying enough nucleotides, proteins, and lipids for a cell to double its total biomass and then divide to produce two daughter cells (Ward and Thompson, 2012). Second, hallmarks of cancer linked to metabolic change (Hanahan and Weinberg, 2011; Ward and Thompson, 2012; Zitvogel et al., 2006). In Hanahan and Weinberg, 2011 review article, they depicted a hypothetical links between different metabolic alterations and the seven nonmetabolic characteristics of neoplasia. These 7 cancer hallmarks can impinge on metabolism. Third, some tumor suppressor proteins are the upstream of some metabolic enzyme. For example, p53 can regulate the glucose transporter GLUT1-4 and involve in pentose phosphate pathway(Levine and Puzio-Kuter, 2010).The research mention above suggested that metabolism of cancer cells is strong coorelated to their biological functions and features which are different from normal cells.

Among all the metabolic difference in cancer cells and normal cells, Warbug effect is the most well-known phenomena which is cancer cells usually use glucose as energy sources but oxidize most pyruvate to lactate (Warburg, 1956). Many studies have indicated that some oncogenic proteins cause Warbug effect like HIF-1α, AKT, Ras etc(Elstrom et al., 2004; Kim and Dang, 2006;

Kim et al., 2006). However, some research discovered that not only glucose but also glutamine is essential for cancer cell metabolism and proliferation(Gaglio et al., 2011; Kung et al., 2011; Lu et al., 2010; Shanware et al., 2011; Turowski et al., 1994). In Lu et al., 2010 preview article, they even created a new conception: Is Glutamine Sweeter than Glucose? They considered that glutamine is more important than glucose because glutamine can not only be the carbon source of energy (glutamiolysis) but also can be the nitrogen source of building bricks of *de novo* synthesis of nucleotides. In addition, some reference pointed out cancer cells can be addicted to glutamine (Medina et al., 1992; Wise and Thompson, 2010). According the reference above, many glutamine related enzymes are the new targets to cancer (2001; Dang, 2011; Gao et al., 2009; Herbst et al., 2008; Kung et al., 2011; Rajagopalan and DeBerardinis, 2011). Taken together, glutamine metabolism may be the target of cancer therapy.

1-3 Metabolomics and cancer

Metabolomics is a systems approach for studying *in vivo* metabolic profiles, which promises to provide information on drug toxicity, disease processes and gene function at several stages in the discovery and development process (Nicholson et al., 2002). To date, metabolomics type studies rely primarily on nuclear magnetic resonance (NMR) or mass spectrometry coupled to chromatography (Dettmer and Hammock, 2004; Robertson et al., 2011). For cancer research field, metabolomics is also the upcoming new science in the omics field with the potential to further increment our knowledge of cancer biology (Claudino et al., 2012). There are more and more research articles use this new method to find out the signatures or biomarker of cancers(Claudino et al., 2012; Dettmer and Hammock, 2004; Herbst et al., 2008; Robertson et al., 2011; Serkova and

Glunde, 2009; Tomita and Kami, 2012).

1-4 Angiopoitin-like proteins and metabolism

Angiogenesis is an important step in embryogenesis, wound healing, and tumorigenesis through the growth of new blood vessels from pre-existing vasculature (Dhanabal et al., 2005). Traditionally, anti-angiogenesis is a pivotal target for cancer therapy. There is a family named angiopoitin family which regulates the angiogenesis. Recently, scientists found that some of angiopoitin family members share the same domains: all possess an N-terminal coiled-coil domain and a C-terminal fibrinogen-like domain, both characteristics of angiopoietins. They named these seven members: Angiopoitin-like proteins 1-7 (ANGPTL 1-7)(Dhanabal et al., 2005; Dhanabal et al., 2002). To date, these seven members are revealed that their original pro-/anti- angiogensis function (Oike et al., 2004; Oike et al., 2003), some papers showed that some of them may have another function. For example, ANGPTLs are found that inhibitory receptors can bind ANGPTLs and support blood stem cells and leukaemia development(Zheng et al., 2012). Recently, ANGPTL4 is considered as an important redox player in cancer and a potential therapeutic target (Zhu et al., 2011). ANGPTL6 increase energy expenditure(Oike et al., 2005). Besides, ANGPTL3 and ANGPTL4 can inhibit LPL activity and increase serum TG levels (Inaba et al., 2003; Le Jan et al., 2003). These data shows that ANGPTLs may possess multi-function including metabolism. So, we want to reveal that if ANGPTL1 shares the same feature of the metabolic regulator like ANGPTL3 and ANGPTL4.

1-5 the FOXOs family and metabolism

FOXO proteins are a subgroup of the Forkhead family of transcription factors. This family is characterized by a conserved DNA-binding domain (the ‘Forkhead box’, or FOX) and comprises more than 100 members in humans, classified from FOXA to FOXR on the basis of sequence similarity. Members of class ‘O’ share the characteristic of being regulated by the insulin/PI3K/Akt and MEK1/Erk signaling pathway (Carter and Brunet, 2007). Among FOXOs family, FOXO3a was shown to be associated with tumour suppression activity and inhibition of FOXO3a expression promotes cell transformation, tumour progression and angiogenesis (Yang et al., 2008). Moreover, FOXO3a is considered as a transcription factor of some metabolic enzymes like PEPCK and NPY. This clue inspired us that FOXO3a may inhibit tumor progression via its ability of transcription which change the level of metabolic enzymes.

1-6 the motivation of this study

Cancer is usually considered as a lethal disease. In this study, we present an anti-angiogenesis factor ANGPTL1 has a new role of regulation function of glutamine catabolism in human lung cancer cell lines. We found that ANGPTL1 may decrease intracellular glutamine level through inducing a metabolic enzyme glutaminase 2 (GLS2) and inhibit cell migration, invasion and anchorage independent ability. We hope we can provide a new point of view to cure lung cancer and prolong the patients’ survivals.

Materials and Methods

2-1 Cell culture

The NSCLC lung cancer cell lines CL1-5, CL1-0, and H1299 were were cultured in DMEM/F12 medium supplemented with 10% FBS at 37°C in a humidified 5% CO_2 atmosphere.CL1-5 and CL1-0 were established in the National Health Research Institutes laboratory and displayed progressively increasing invasiveness. H1299 was obtained from the American Type Culture Collection (ATCC).

2-2 Immunohistochemistry

Clinical patients' tissues were obtained from National Taiwan University Hospital. Written informed consent was obtained from all the patients. After rehydration, 4μm sections of paraffin-embedded tissue on glass slides were incubated in 3% hydrogen peroxide to block the endogenous peroxidase activity. After trypsinization, sections were blocked by incubation in 3% bovine serum albumin in PBST. The primary antibody, a polyclone rabbit anti-human GLS2 antibody (ABGENT) was added to the slides at a dilution of 1:50 (diluted in 3% bovine serum albumin) and incubated at 4 °C overnight. After three times washing in PBST, the slides were treated with goat anti-rabbit IgG biotin labeled secondary antibodies (Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature. Conjugated antibodies were detected with a DAB kit (Vector Laboratories). The slides were stained with diaminobenzidine, and then washed, counterstained with Delafield's hematoxylin. The following steps are dehydrated, treated with

xylene, and mounted. A scoring system was devised to assign a staining intensity score for GLS2 expression from 0 (no expression) to 3 (highest intensity staining). Immunostaining was classified into one of two groups according to both intensity and extent: low expression was defined as no staining present (staining intensity score=0) or equal to 10% of the cells (staining intensity score =1); high expression was defined as positive immunostaining present 10%–50% of the cells (staining intensity score =2) or more than 50% of the cells (staining intensity score =3).

2-3 Western blotting analysis

Cells were scraped with scrapers and then lysed with RIPA buffer (50 mM Tris-HCl, pH7.4, 150 mM NaCl, 1% Trinton X-100, 10% glycerol, 1 mM EDTA, 1 mM Na3VO4, 1 mM NaF, 1mM PMSF, 1μg/ml leupetin, and aprotinin), centrifuged with the speed of 14000 rpm for 20 min at 4℃, and then separated by 10% SDS-polyacrylamide gel electrophoresis. The proteins were transferred to polyvinylidene difluoride (PVDF) Immoilon membrane (Bio-Rad,Hercules, CA). The membranes were blocked with 10% milk before reacted with primary antibodies for one night. After washing in PBST buffer, membranes were reacted with appropriated secondary antibodies conjugated with horseradish peroxidase (HRP) in 5% milk. Bound antibodies on the membranes were detected using an enhanced chemiluminescence (ECL) detection system according to the manufacturer's manual (AMersham Biosciences).

2-4 Transfection and Lentivirus infection

Transfections were performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacture's instruction. The GLS1, GLS2 and Foxo3A shRNAs were purchased from National RNAi Core Facility in Academic Sinica, Taipei, Taiwan. Lentiviruses were produced by co-transfecting shRNA-expressing vector, packaging plasmid pCMV-ΔR8.91 (containing *gag*, *pol* and *rev* genes) and envelope plasmid pMD.G (VSV-G expressing plasmid) into 293T cells by using calcium chloride. Viral supernatants were collected and used to infect cells with 8 μg/mL polybrene. Stable expressing cell clones were selected by using 2 μg/mL puromycin.

2-5 MTT proliferation assay

The cell proliferation kit (MTT, Roche Diagnostics, Mannheim, Germany) was performed in 24-well format according to the manufacturer’s instructions. Cells (2 x 10^4 cells) were seeded in 24-well plates. The cell growth rate were determined by using MTT (3-(4,5-Dimethy-lthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) assay, a method applying the mitochondrial metabolic enzyme activity as an indicator of cell viability. The formazan crystals resulting from mitochondrial enzymatic activity on the MTT substrate were solubilized with 500 μl of dimethyl sulfoxide. 150 μl of solution was added into 96-well plate. The light absorbance was measured at 570 nm with spectrophotometer. Each individual experiment was repeated at least 3 times.

2-6 RNA isolation and reverse transcription-polymerase chain reaction

Total RNA was extracted via TRizol (Invitrogen) according to the manufacturer's instructions. cDNA synthesis was performed with 2 μg of each sample using the SuperScript® III first strand synthesis kit (Invitrogen) according to the manufacturer's instructions. The cDNAs were amplified with the forward (F) and reverse (R) primers by polymerase chain reaction (PCR) as described. The PCR amplification was carried out in a reaction buffer containing 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, all four dNTPs (each at 6.25 μM), 2.5 U of *Taq* DNA polymerase, and 0.5 μM primers. The reactions were performed in a Biometra Thermoblock (Biometra, Hamburg, Germany) with the following program: denaturing for 30 seconds at 95 °C, annealing for 40 seconds at 55 °C (58 °C for GLS2 in CL1-5, 55 °C for H1299), and elongating for 30 seconds at 72 °C, for a total of 32 cycles (26 cycle for GAPDH, 36 cycle for GLS2 in both CL1-5 and H1299); the final extension took place at 72 °C for 10 minutes. Equal volumes of each PCR sample were separated by electrophoresis in 1.5 % agarose gel, which was then visualized with ethidium bromide and photographed under UV excitation.

2-7 Mouse model for tumorigenesis

All animal works were done in accordance with a protocol approved by the National Taiwan University College of Medicine and National Taiwan University College of Public Health institutional animal care and use committees. Age-matched nonobese diabetic severe combined immunodeficient (SCID) male mice (6–8 weeks old) were used. For orthotopic metastasis assays, cells (5×10^5 H1299 cells) were resuspended in a 1:1 mixture of PBS and GFR-Matrigel (BD

Labware Bedford, MA, USA). This mixture was then injected into the left lateral thorax of each mouse. Metastatic nodules in the right lung were quantified using a dissecting microscope at each end point. This orthotopic model of lung cancer is referred to (Chen et al., 2005). If the cells can be found in right lung, this phenomenon is considered as metastasis.

2-8 Anchorage-independent growth assay

The anchorage-independent growth ability was determined by soft agar assay. The cell-growth matrix was consisted of base and top agarose in 24-well culture plates. The base layer contained DMEM/F12 medium, 0.5% agarose, and10% FBS. The top layer contained DMEM/F12 medium, 0.35% agarose, 10% FBS and cells. These plates were incubated at the 37°C in a humidified 5% CO_2 atmosphere for 2-3 weeks. During these 2-3 weeks, about 40-50 μL medium was added into these gels to prevent from drying.

2-9 Invasion and migration ability assay

Invasion and migration assays were performed by using transwell inserts for a 24-well plate containing 8 μm pores (Millipore, Bedford, MA, USA). Matrigel (BD Labware, Bedford, MA, USA) coated filters were used for the invasion assay. Cells were seeded in the upper chamber for 24 hours. Cells were fixed with methanol for 10 minutes and stained with crystal violet, and then counted under the microscope. The number of cells that migrated was normalized to the growth rate for each

cell line with MTT assay.

2-10 Metabolomic samples preparation

Cells ($1x10^6$) were seeded over night in 6 cm dish and then wash twice with cold PBS. After scraping into eppendorfs, we add 1 ml 50% methanol to each eppendorf to extract the water-soluble metabolites. After centrifugation, the supernatant was collected as water-soluble metabolites.. Further, we add chloroform to extract lipid-soluble the metabolites. After centrifugation, the supernatant was collected as lipid-soluble metabolites. The water-soluble and lipid-soluble metabolites are dried by nitrogen gas in hood. All extraction samples are stored at -80℃ freezer before LC/MS analysis. The water-soluble samples are resolved in acetonitrile and then inject into the LC/MS. The operation of LC/MS, measurement the level of intracellular metabolites and interpretation of these LC/MS peaks were done by metabolomics group of Dr. Yufeng Jane Tseng and Dr. Ching-Hua Kuo.

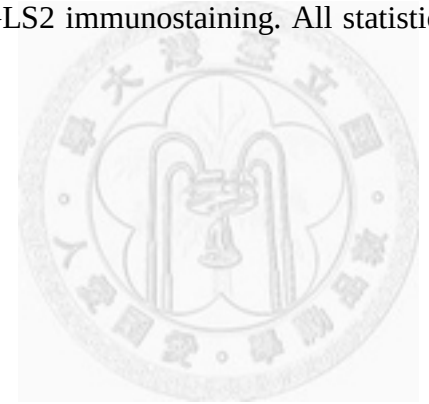
2-11 Principal componment analysis (PCA), LC/MS and metabolomic analysis

Principal component analysis (PCA) was performed using the significant variables. PCA is a data reduction technique that aims to identify patterns and similarity among data samples. PCA uses a linear transformation to convert data into new variables called principal components (PC) with each PC orthogonal and uncorrelated to each other. The first PC captures the most variability in the data,

and each successive PC has the highest variance unexplained by the preceding PCs.

2-12 Statistical Analysis

All observations were confirmed by at least three independent experiments. The data were presented as mean ± SD. ANOVA was used to evaluate the statistical significance of the mean values. Cox proportional hazards regression was used to test the prognostic significance of factors in univariate and multivariate models. Spearman's rank correlations were determined for comparison of ANGPTL1 and GLS2 immunostaining. All statistical tests were two-sided, and $P < 0.05$ was considered significant.



Results

3-1 ANGPTL1 induced GLS2 altered the glutamine level in lung cancer cell lines

To determine what kind of metabolic pathway which ANGPTL1 may participates in, we use metabolomic methods to reveal what kinds of small molecular metabolites have been changed. In PCA analysis showed a good separation between the CL1-5/neo and CL1-5/ANGPTL1. Principal component 1 (PC1) accounted for a high proportion (74.47%) of the total variance, while principal component 2 (PC2) was attributable to only 11.83% of the total variability (Fig.1a). To further investigating what kind of metabolites were changed, we use bioinformatics methods to determine these small molecular. We found that there are several metabolites including theronine, phenylpyruvate, hypoxanthine, spermidine, 2-phenylacetamide, methionine, tyrosine, UDP-N-acetyl-glucosamine, and glutamine were downregulated and creatine was upregulated in CL1-5/ANGPTL1 which p-value was less than 10^{-10} (Fig. 1b). Among these metabolites, though L-glutamine reduced only about 33% compared to other metabolites change level is not much, according to some research, these other metabolites seems to be the downstream metabolites of glutamine. So, we propose that ANGPTL1 may change glutamine metabolism by change the level of glutamine-related enzymes. Here we use RT-PCR technology to confirm our hypothesis. We check some glutamine-related enzymes including glutamine sythetase (GS), glutaminase 1(GLS1), glutaminase2 (GLS2) these 3 main metabolic enzymes. We can find that only GLS2 was increased in overxepression ANGPTL1 cells (Fig. 1c).

According to some studies, GLS2 has been reported for its hydrotase activity. Here, we use RNA interference technology to knockdown the GLS2 mRNA (Fig. 1d). We found that the level of glutamine in cells can be restored after GLS2 mRNA was inhibited (Fig. 1e). These data show that

ANGPTL1 may regulate glutamine level in lung cancer cell line through increasing GLS2, which has been reported as a tumor suppressor. The data above shows that overexpression ANGPTL1 upregulated GLS2 and caused intracellular glutamine level decreased.

3-2 ANGPTL1- induced GLS2 did not affect cell proliferation but invasion, migration and anchorage independent ability

In order to investigate the role of ANGPTL1-induced GLS2, we use RNA interference technology to knockdown GLS2 RNA. According to the cancer cells' glutamine addiction, we further analyze the glutamine level of CL1-0 and CL1-5 (Fig. 2a). We proposed that glutamine metabolism may change the cell mobility. So we tried to test the invasion and migration ability in overexpression ANGPTL1 and further knockdown GLS2 in CL1-5 and H1299. We found that knockdown GLS2 can increase invasion and migration ability in CL1-5/ANGPTL1 and H1299/ANGPTL1 cells (Fig. 2b, Fig. 2c). However, cell proliferation rate in CL1-5 and H1299 cells did not change (Fig. 2d, Fig. 2e). Although there is another isozyme named GLS1 which are two main hydrotases of glutamine, we can find that GLS1 mRNA level did not change in CL1-5/ANGPTL1 (Fig. 1c). However, GLS1 and GLS2 are isozymes which mean their function is the same. So, maybe when we knockdown GLS2 mRNA, cells will compensate back by increasing GLS1. Here, we also knockdown GLS1 to test if the level of GLS1 and GLS2 were changed due to glutamine level change. We found that knockdown GLS1 did not change GLS2 level and the ANGPTL1-induced invasion migration ability (Fig. 2f). Then, knockdown GLS2 not only can promote cells' mobility, but can increase the anchorage independent ability. We found that anchorage independent ability can be inhibited by overexpression ANGPTL1 and regain via knockdown GLS2 in H1299 cell (Fig. 2g, Fig. 2h). The

total cell numbers can be observed decreased in overexpression ANGPTL1 and increased in H1299/ANGPTL1/sh-GLS2.These data show that ANGPTL1 would affect lung cancer cells' progression including invasion ability, migration ability and anchorage independent ability.

3-3 ANGPTL- induced GLS2 changed the lung cancer cell progression *in vivo*

To further investigate ANGPTL1-induce GLS2 may change cancer progression *in vivo*; we use 6-week old SCID male mice to test if knockdown GLS2 may alter lung caner cell line H1299 via orthotopic metastasis assays. We found that tumor size is quite different among control group, overexpression ANGPTL1 group and knockdown ANGPTL1-induced GLS2 group after 4 weeks (Fig. 3a). In control group, primary tumor volume was bigger than overexpression ANGPTL1 group. In knockdown GLS2 group, primary tumor sometimes even bigger than control group. Moreover, ANGPTL1 inhibited lung metastasis but in GLS2 knockdown group, metastasis can be observed (Fig. 3a). We also found the nodule numbers in control group were more than overexpression ANGPTL1 (Fig. 3b). These data suggested that ANGPTL1 can inhibit lung cancer cells metastasis via GLS2 expression.

3-4 GLS2 was induced by ANGPTL1 through MEK1-FOXO3A signaling

Because the mechanism of how ANGPTL1 can affect metabolic enzymes is not clear, we want to investigate the pathways of ANGPTL1-induced GLS2. First, we found that the p-Akt and p-Erk are

decreased in CL1-5/ANGPTL1 compared to CL1-5/neo (Fig. 4a). Here, we propose that ANGPTL1 may affect metabolic enzymes through these signal pathways because ANGPTL1 is a secreted ligand which can bind to membrane receptor(s) and activate (or shot down) a series of signal transduction to change GLS2 gene expression. Because GLS2 can be regulated at RNA level, maybe GLS2 can be regulated by some transcription factors through a series of signaling pathways. Second, we search the upstream of GLS2 gene. We found that there are 2 consensus sequences in the GLS2 promoter region (Fig. 4b). These 2 consensus sequences may be recognized by a transcription factor family named FOXO family. Third, according to some research, when FOXOs can be phosphorylated by p-Akt or/and p-Erk, FOXOs' transcription activity are turned off. Here, we porposed that ANGPTL1 can upregulate GLS2 via inhibition of p-Akt or/and p-Erk which can not phosphorylate FOXOs. So, we started with transfection technology to transfect myr-Akt and MEK1 plasmid into CL1-5/neo and CL1-5/ANGPTL1 cells. We found that after transfecting myr-Akt and MEK1, GLS2 expression was downregulated in CL1-5/ANGPTL1 when we transfect MEK1 (Fig. 4d) but GLS2 expression did not change significantly when we transfect myr-Akt (Fig. 4c). So, we comfirmed the results of GLS2 expression may related to MEK1-Erk pathway. Further, we tried to investigate FOXOs family members: FOXO1 and FOXO3a via western blot and found that only FOXO3a can be upregulated (Fig. 4e). This data showed that FOXO3a may be the transcription factor of GLS2. Then, we further confirmed after transfecting myr-Akt and MEK1plasimd. We found that level of FOXO3a was downregulated in transfecting MEK1 group but not transfecting myr-Akt group in CL1-5/ANGPTL1 (Fig. 4f, Fig. 4g). In addition, we use sh-RNA to knockdown FOXO3a in CL1-5/ANGPTL1. We found that the level of GLS2 can be downregulated (Fig. 4h). These data suggested that ANGPTL1 induces GLS2 via downregulation of p-Erk which caused FOXO3a can not be phosphorylated. If FOXO3a can not be phosphorylated, FOXO3a will stay in nucleus and bind to GLS2 promoter region and cause GLS2 mRNA expression.

3-5 The clinical correlation between ANGPTL1 and GLS2

To elucidate the clinical relevance of ANGPTL1 and GLS2 in cancer patients, we analyzed 82 lung cancer specimens using immunohistochemical analysis (IHC) with GLS2-specific antibody. Collectively, GLS2 was expressed at a high level in normal lung tissues and preferentially expressed in lung tumor tissues (Fig. 5a-f). The prognostic significance of ANGPTL1 and GLS2 expression was determined by assessing its nuclear staining using these 82 human lung cancer specimens with known clinical follow-up records. Figure 5a-f shows representative examples with different GLS2 scores in normal parts and tumor parts. Among these specimens, we found that high GLS2 expression level (scores of 2 and 3) correlated strongly with increased overall survival relative to tumors with low GLS2 expression level (scores of 0 and 1) as shown in Fig. 5g. Similar results were obtained for disease-free interval (Fig. 5g). Theseverity of tumor grade, the presence of ANGPTL1,the presence of GLS2, the stage of the disease and the status of metastasis were assessed by Cox regression models to predict the clinical outcome. Univariate analysis revealed that the presence of ANGPTL1 expression, GLS2 expression, the stage of the disease and lymph nodal status were significantly associated with overall survival (Harzard ratio = 0.5515, 0.4119, 2.0119, 2.0451; P = 0.03346, 0.002812, 0.009252, 0.005089, respectively). Backward stepwise multivariate analysis revealed that the presence of GLS2 and pathological stage were independent risk factors associated overall survival (Hazard ratio = 0.3262, 2.4770; P = 0.000531, 0.01612 respectively; Table 1). Next, we want to reveal that the correlation between ANGPTL1 and GLS2 is not only in cells but also in clinical patients. We investigated whether ANGPTL1 expression positively correlated with GLS2 levels in human lung cancer patients. The representative of high expression and low expression of ANGPTL and GLS2 were showed in Fig 5h. Immunohistochemistry analysis

of lung cancer specimens revealed a positive correlation between ANGPTL1 and GLS2 expression (tested by Spearman's nonparametric correlation test, correlation coefficient = 0.294, *P* =0.0081; Table 2). These data show that ANGPTL1 is positively correlated to GLS2 and these 2 protein molecules are both correlated to survival in clinics.

Discussion

It has been known for a long time that cancer cells have elevated aerobic glycolysis (the Warburg effect) and exhibit increased dependence on glutamine for growth and proliferation (Lu et al., 2010). In recent study, transformed cells exhibit a high rate of glutamine consumption that cannot be explained by the nitrogen demand imposed by nucleotide synthesis or maintenance of nonessential amino acid pools. Rather, glutamine metabolism provides a carbon source that facilitates the cell's ability to use glucose-derived carbon and TCA cycle intermediates as biosynthetic precursors (DeBerardinis et al., 2007). In other study, B cell can use glucose-independent glutamine metabolism via TCA cycling for proliferation and survival (Le et al., 2012). These research show that glutamine uptake is benefit to tumor growth. However, in clinical patients, doctors usually suggest cancer patients to uptake some glutamine after surgery (Agostini and Biolo, 2010; Fan et al., 2009; Furukawa et al., 2000). This glutamine uptake paradox seems to need to investigate that if the role of glutamine is benefit to cancer progression. In our study, GLS2 hydrolyzes the glutamine into glutamate and glutamate can further be converted to α-ketoglutarate may increase the TCA cycle to against Warburg effect. This may be the reason why GLS2 can change the tumor progression.

Two recent studies showed that the tumor suppressor p53 could transcriptionally activate the liver type glutaminase (GLS2), promoting glutamine metabolism and glutathione generation (Hu et al., 2010; Suzuki et al., 2010). In normal part of liver, the level of GLS2 is more than the tumor part of liver. These articles suggested that GLS2 possess a role of tumor repressor. Another studies about glutaminase1 (GLS1) which is regulated by an oncogene Myc (Gao et al., 2009; Le et al., 2012) show that inhibition of GLS1 is a target to block cancer cell growth. However, GLS1 and GLS2 are isozyme which hydrolyzes the amino group of glutamine. Interestingly, these two enzymes execute the same function but cause totally different results. In (Levine and Puzio-Kuter, 2010), they asked this question: these two glutaminases, regulated by myc (glutaminase 1) and p53 (glutaminase 2),

have opposite effects on the cell remains to be elucidated. In our study, we found that GLS1 and GLS2 are independent. According to (Suzuki et al., 2010), GLS2 inhibits tumor cell growth and colony formation and GLS2 expression is decreased in liver tumors. However, in our study, GLS2 which is induced by ANGPTL1 did not change the growth rate of cell. It may be explained by our GLS2 is induced by ANGPTL1. This expression method is different from Suzuki et al., 2010. However, GLS2 function is different within different expression method, but taken together, GLS2 is a tumor suppressor.

In (Nicklin et al., 2009) research, they find that certain tumor cell lines with high basal cellular levels of L-glutamine bypass the need for L-glutamine uptake and are primed for mTOR activation. They think L-glutamine flux regulates mTOR, translation and autophagy to coordinate cell growth and proliferation. In our study, we did not find autophagy in ANGPTL1 overexpression cell although the glutamine in CL1-5/neo is higher. This needs more research on the autophagy and mTOR pathway to reveal that CL1-5/ANGPTL1 is not undergoing autophagy. Another interesting issue about GLS2 is the research in (Olalla et al., 2002), they mention that GLS2 was localized in nuclei. But in our IHC staining, we did not observe GLS2 was localized in nuclei. Moreover, we found that GLS2 is induced by ANGPTL1-p-Erk-FOXO3a signaling pathway. Our data shows that under ANGPTL1 regulation, GLS2 may not localize in nuclei although GLS2 process the ankyrin domain but in mitochondria to hydrolyze glutamine.

In conclusion, we present a role of ANGPTL1, which is considered as an angiogenesis-related protein; have a novel function that inhibit tumor progression via inducing GLS2 expression and changing glutamine metabolism. ANGPTL1 and GLS2 are tumor suppressors *in vitro*, *in vivo* and in clinical patients. Further, these 2 proteins may contribute to lung cancer therapy and inhibit lung cancer progression.

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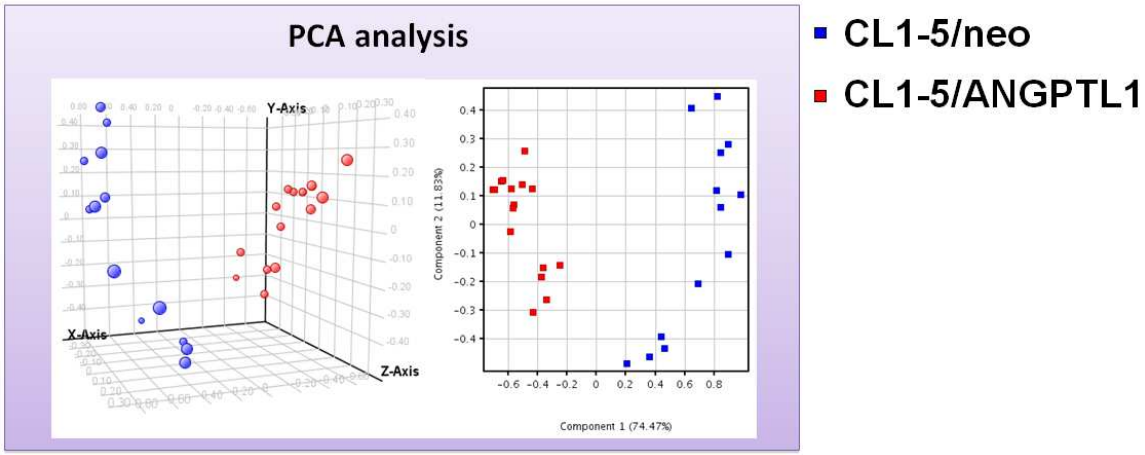
Figures and figure legends

Fig. 1a



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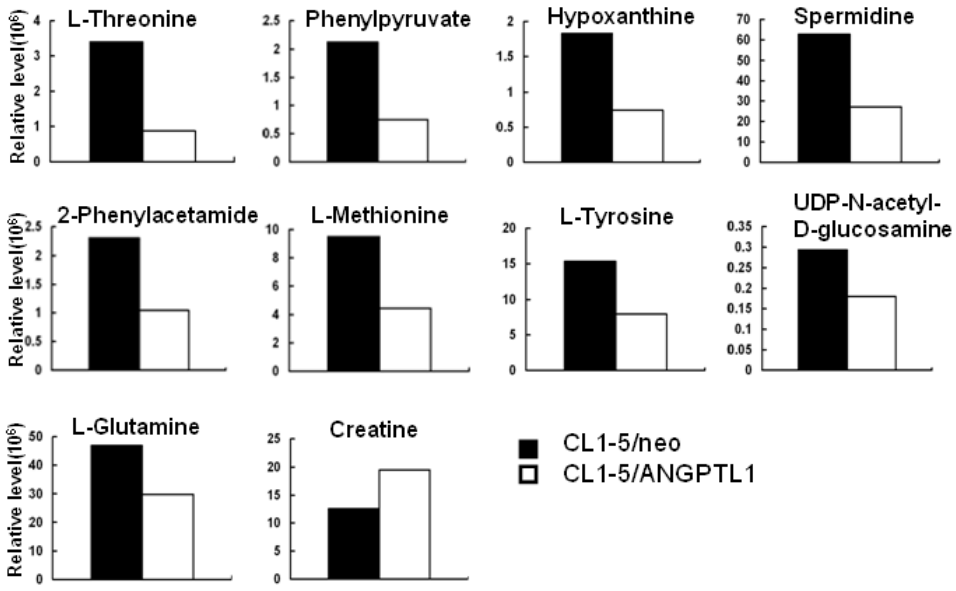

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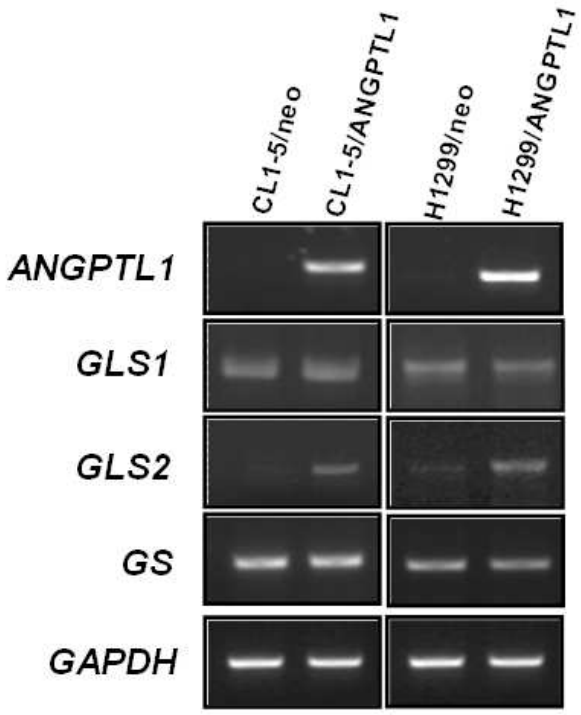
CL1-5/neo
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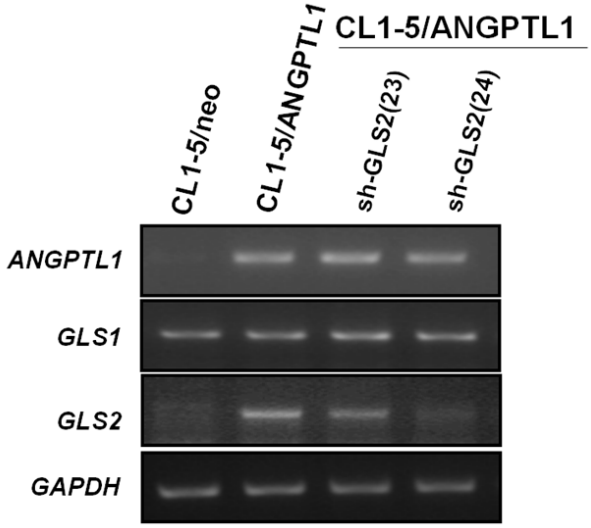
GLS1
GLS2
GS
GAPDH

Fig. 1d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CL1-5/neo
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sh-GLS2(23)
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ANGPTL1
GLS1
GLS2
GAPDH

Fig. 1e

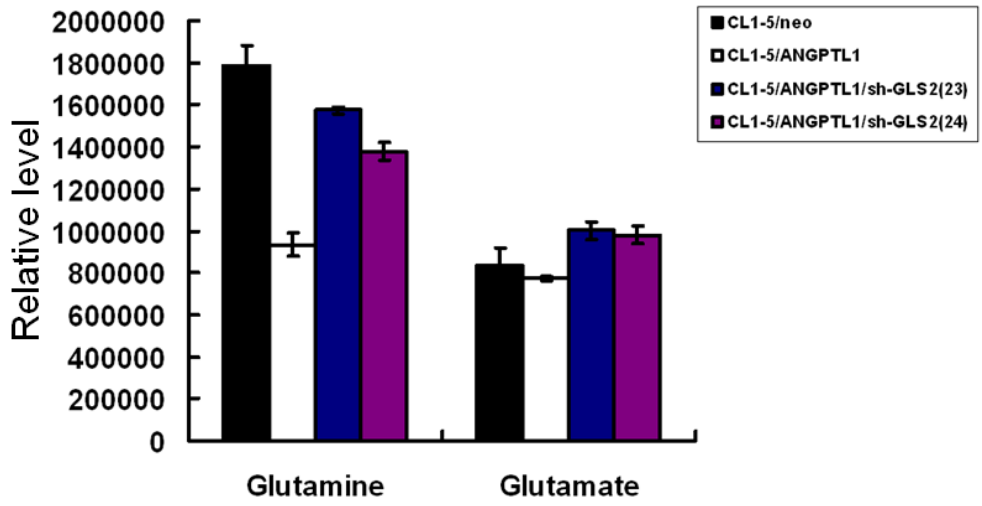

Fig. 1 metabolomic profile and glutamine metabolism is changed by glutaminase 2.
(a) the PCA analysis of CL1-5/neo and CL1-5/ANGPTL1. (b) The metabolites which are significantly change in CL1-5/neo and CL1-5/ANGPTL1. (c) RT-PCR results of glutamine related enzymes. (d) the PCR results of CL1-5/neo, CL1-5/ANGPTL1 and CL1-5/ANGPTL1/sh-GLS2. (e) the level of intracellular glutamine and glutamate in CL1-5/neo, CL1-5/ANGPTL1, and CL1-5/ANGPTL1 knockdown GLS2 cells.

Fig. 2a

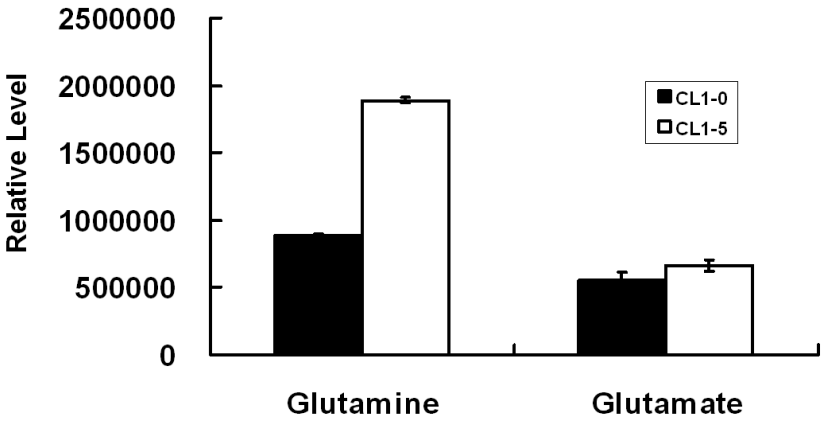
Relative Level
2500000
2000000
1500000
1000000
500000
0
CL1-0
CL1-5
Glutamine
Glutamate

Fig. 2b

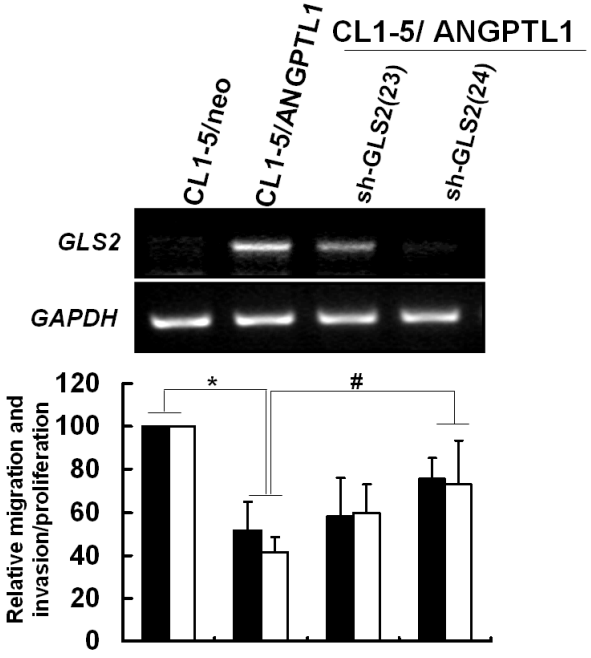
CL1-5/ ANGPTL1
CL1-5/neo
CL1-5/ANGPTL1
sh-GLS2(23)
sh-GLS2(24)
GLS2
GAPDH
Relative migration and invasion/proliferation
120
100
80
60
40
20
0
*
#

Fig. 2c

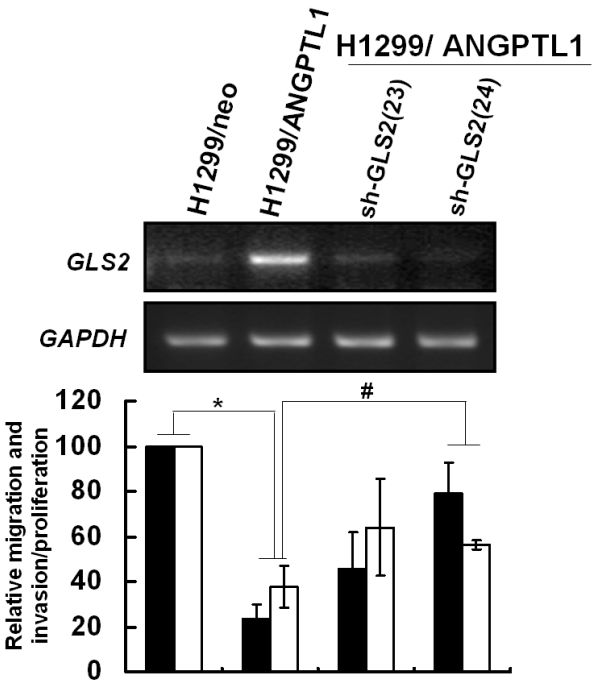
H1299/ ANGPTL1
H1299/neo
H1299/ANGPTL1
sh-GLS2(23)
sh-GLS2(24)
GLS2
GAPDH
Relative migration and invasion/proliferation
120
100
80
60
40
20
0
*
#

Fig. 2d

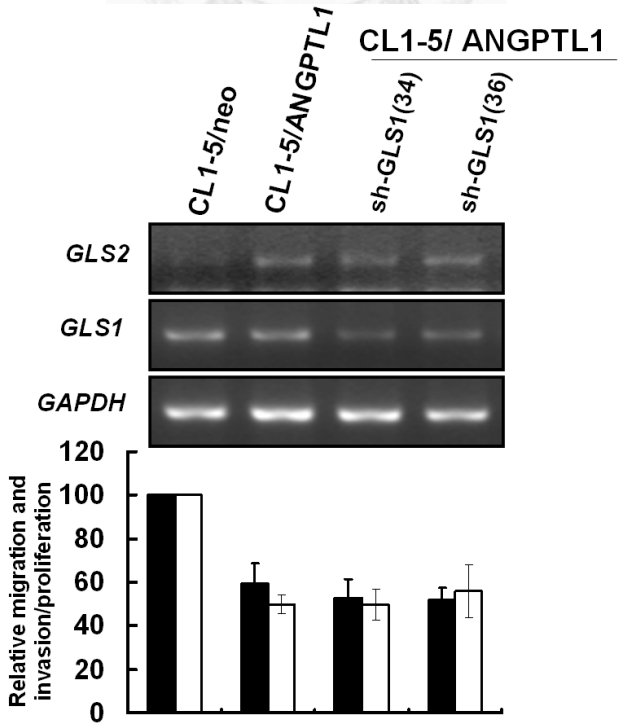
CL1-5/ ANGPTL1
CL1-5/neo
CL1-5/ANGPTL1
sh-GLS1(34)
sh-GLS1(36)
GLS2
GLS1
GAPDH
Relative migration and invasion/proliferation
120
100
80
60
40
20
0

Fig. 2e

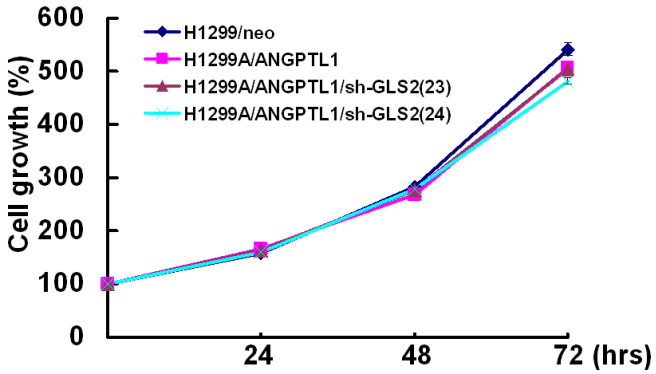
600
500
400
300
200
100
0
Cell growth (%)
H1299/neo
H1299A/ANGPTL1
H1299A/ANGPTL1/sh-GLS2(23)
H1299A/ANGPTL1/sh-GLS2(24)
24
48
72 (hrs)

Fig. 2f

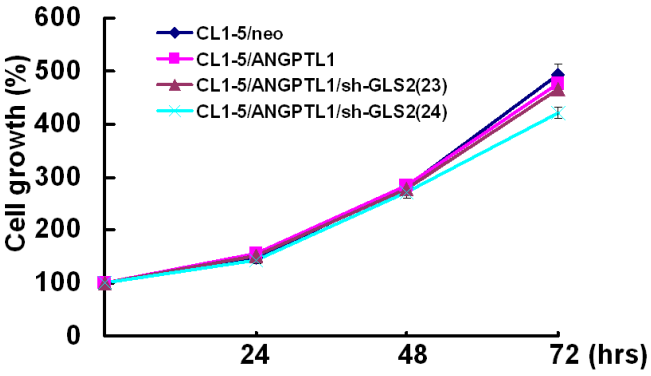
600
500
400
300
200
100
0
Cell growth (%)
CL1-5/neo
CL1-5/ANGPTL1
CL1-5/ANGPTL1/sh-GLS2(23)
CL1-5/ANGPTL1/sh-GLS2(24)
24
48
72 (hrs)

Fig. 2g

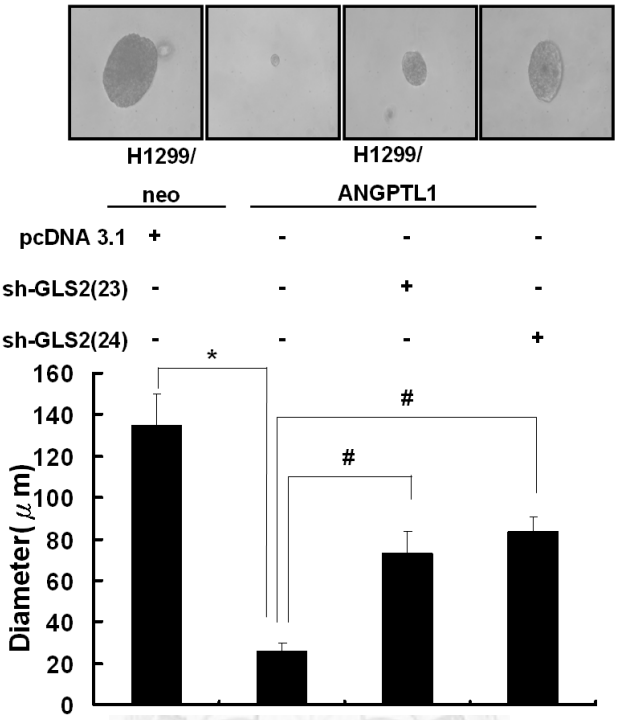
H1299/
neo
H1299/
ANGPTL1
pcDNA 3.1 + - - -
sh-GLS2(23) - - + -
sh-GLS2(24) - - - +
Diameter(μm)
0
20
40
60
80
100
120
140
160
*
#
#

Fig. 2h

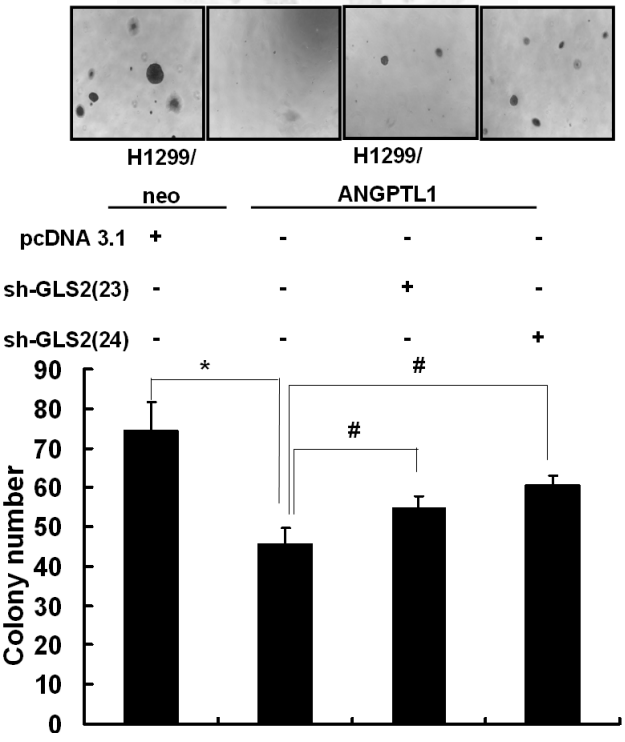
H1299/
neo
H1299/
ANGPTL1
pcDNA 3.1 + - - -
sh-GLS2(23) - - + -
sh-GLS2(24) - - - +
Colony number
0
10
20
30
40
50
60
70
80
90
*
#
#

Fig. 2 ANGPTL1 inhibited the invasion, migration, and anchorage independent ability in lung cancer cells via inducing GLS2.

(a) The level of intracellular glutamine in CL1-0 and CL1-5. (b) Invasion and migration ability in CL1-5/neo, CL1-5/ANGPTL1, CL1-5/ANGPTL1/sh-GLS2 (23) CL1-5/ANGPTL1/sh-GLS2 (24). (c) Invasion and migration ability in H1299/neo, H1299/ANGPTL1, H1299/ANGPTL1/sh-GLS2 (23) and H1299/ANGPTL1/sh-GLS2 (24). (d) Invasion and migration ability in CL1-5/neo, CL1-5/ANGPTL1, CL1-5/ANGPTL1/sh-GLS1 (34) CL1-5/ANGPTL1/sh-GLS2 (36). (e)(f) MTT assay in overexpression ANGPTL1 and knockdown ANGPTL1-induced GLS2 in CL1-5 and H1299 cell. (g)(h) Colony size and numbers of anchorage independent assay in H1299 lung cancer cell line. *, #: statistically significant difference ($P < 0.05$)

Fig. 3a

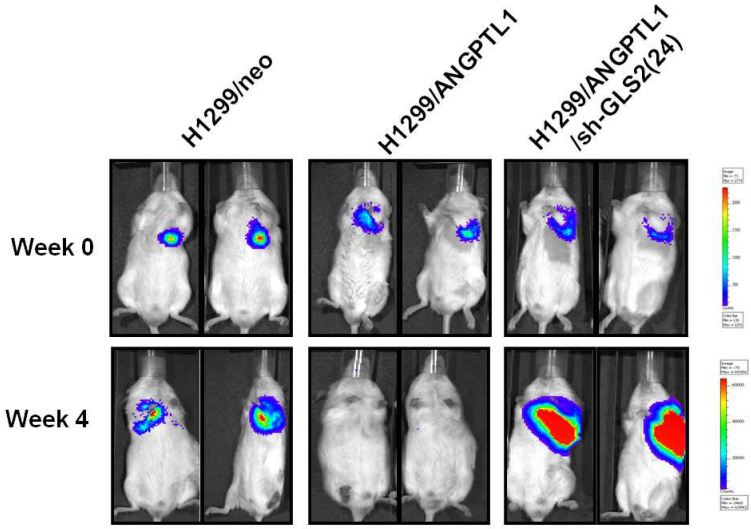
H1299/neo
H1299/ANGPTL1
H1299/ANGPTL1
/sh-GLS2(24)
Week 0
Week 4

Fig. 3b

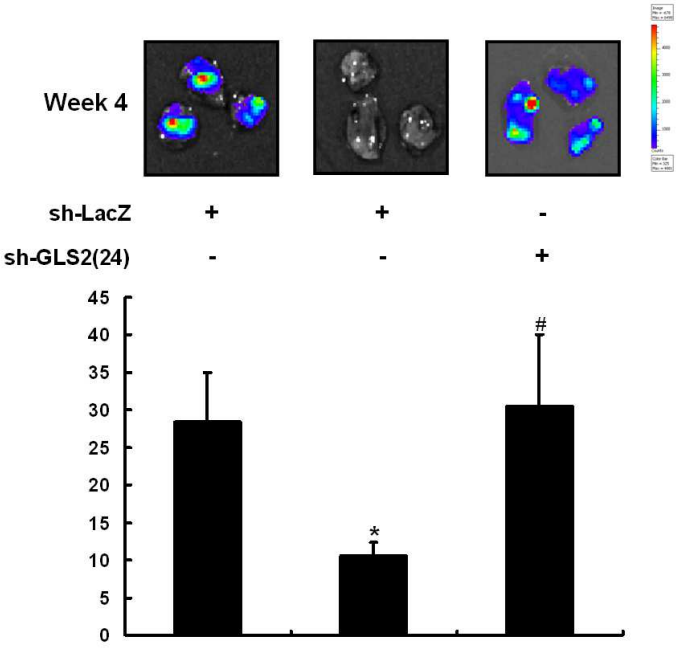
Week 4
sh-LacZ + + -
sh-GLS2(24) - - +
45
40
35
30
25
20
15
10
5
0
*
#

Fig. 3 ANGPTL1-induced GLS2 changes the cell mobility and tumorgenisity *in vivo*.
The luciferase activities of these clones were performed by IVIS system after excitation.
(a) Growth pattern of xenograft tumors formed by H1299 control, H1299/ANGPTL1 and GLS2 knockdown cells. Bioluminescence signals were measured once a week and at endpoint respectively. Photons emitted from specific regions were quantified using Living Image® software (Xenogen Corporation). In vivo luciferase activity is expressed as photons/second/cm^2. (b) The nodule numbers are counted after sacrifice
*: statistically significant difference ($P < 0.05$), as compared with H1299/neo group.
#: statistically significant difference ($P < 0.05$), as compared with H1299/ANGPTL1 group.

Fig. 4a

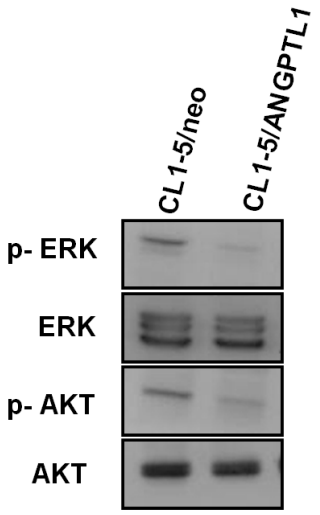
CL1-5/neo
CL1-5/ANGPTL1
p- ERK
ERK
p- AKT
AKT

Fig. 4b

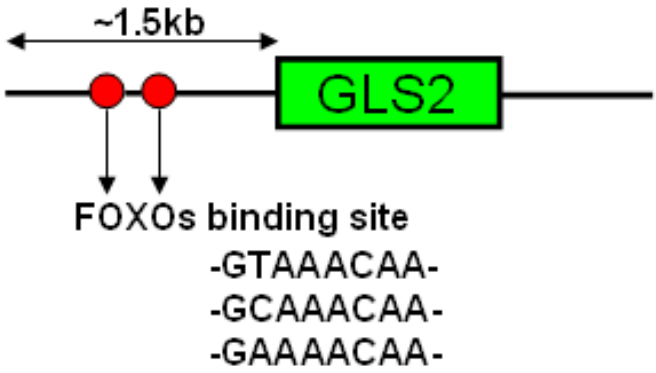
~1.5kb
GLS2
FOXOs binding site
-GTAAACAA-
-GCAAACAA-
-GAAAACAA-

Fig. 4c

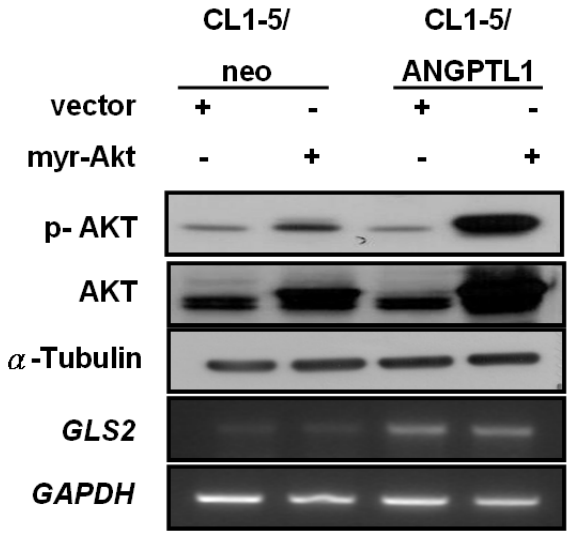
CL1-5/
neo
CL1-5/
ANGPTL1
vector
+
-
+
-
myr-Akt
-
+
-
+
p- AKT
AKT
α-Tubulin
GLS2
GAPDH

Fig. 4d

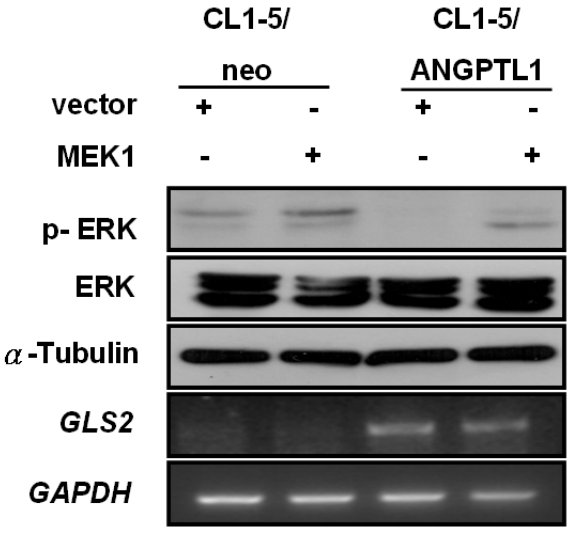
CL1-5/
neo
CL1-5/
ANGPTL1
vector
+
-
+
-
MEK1
-
+
-
+
p- ERK
ERK
α-Tubulin
GLS2
GAPDH

Fig. 4e

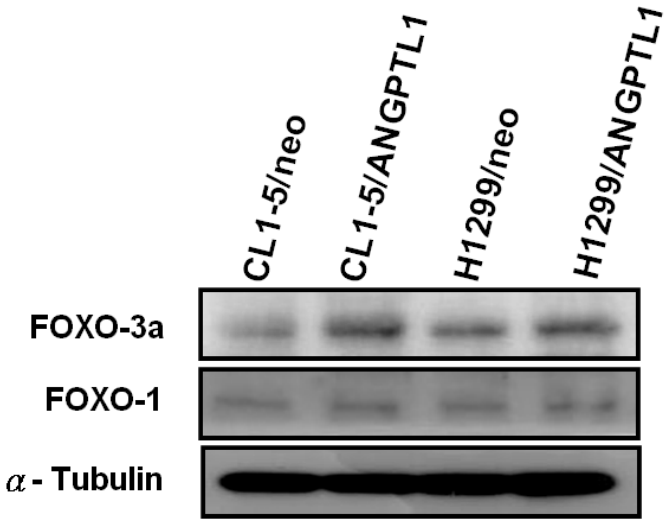
CL1-5/neo
CL1-5/ANGPTL1
H1299/neo
H1299/ANGPTL1
FOXO-3a
FOXO-1
α - Tubulin

Fig. 4f

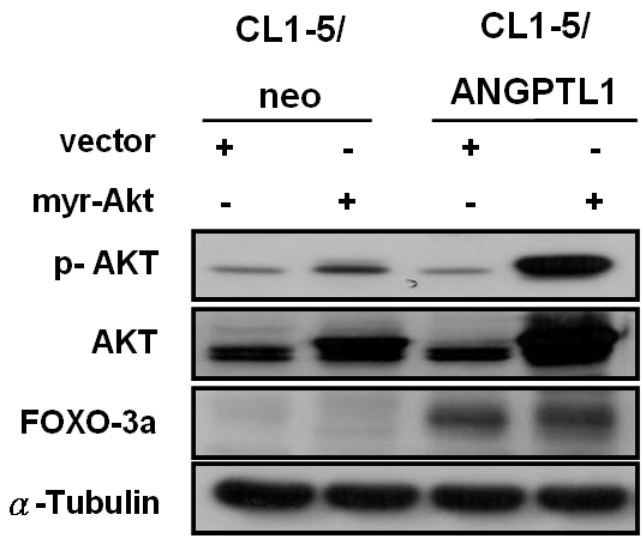
CL1-5/ neo
CL1-5/ ANGPTL1
vector + - + -
myr-Akt - + - +
p- AKT
AKT
FOXO-3a
α -Tubulin

Fig. 4g

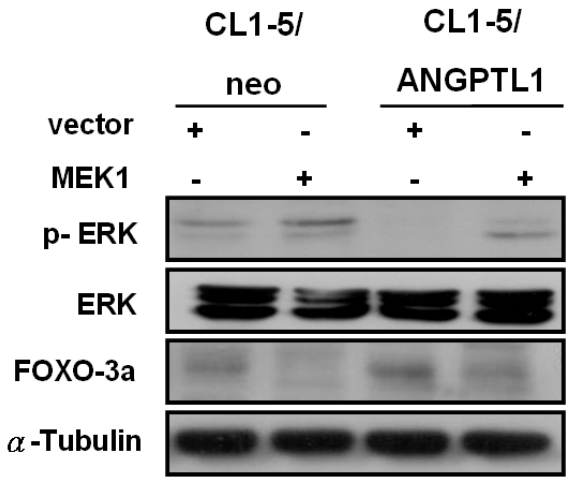
CL1-5/
neo
CL1-5/
ANGPTL1
vector
+
-
+
-
MEK1
-
+
-
+
p- ERK
ERK
FOXO-3a
α-Tubulin

Fig. 4h

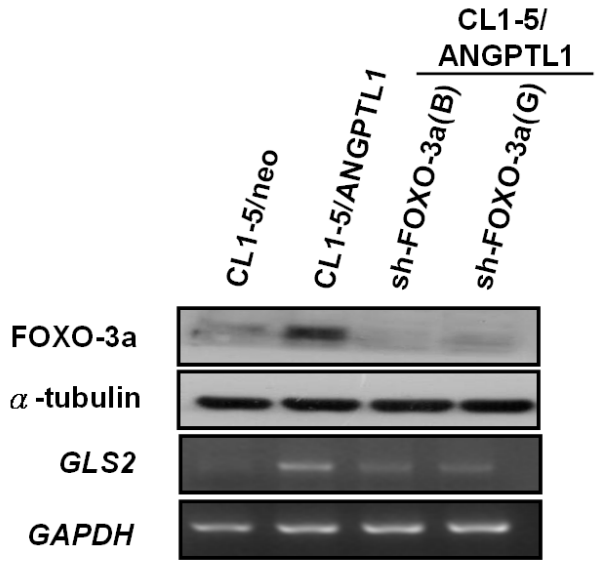
CL1-5/
ANGPTL1
CL1-5/neo
CL1-5/ANGPTL1
sh-FOXO-3a(B)
sh-FOXO-3a(G)
FOXO-3a
α-tubulin
GLS2
GAPDH

Fig. 4 Signal transduction mechanism involved in ANGPTL1 mediated GLS2 upregulation in CL1-5 lung cancer cell.

(a) The level of p-Akt and p-Erk in control and CL1-5/ANGPTL1 group via western blots. (b) At the GLS2 promoter region (~1.5kb upstream of GLS2 gene), there are 2 cognate recognition sites of FOXOs family. (c) The level of GLS2 after transfection of myr-Akt plasmid 72 hrs in CL1-5/neo and CL1-5/ANGPTL1. (d) The level of GLS2 in transfection of MEK1 plasmid 72 hrs in CL1-5/neo and CL1-5/ANGPTL1. (e) The level of FOXO3a and FOXO1 in CL1-5/neo nad CL1-5/ANGPTL1. (f) The western blots of FOXO3a after transfection of myr-Akt plasmid 72hrs. (g) After transfection of MEK1 plasmid 72hrs, the level of FOXO3a was presented via western blots. (h) The level of GLS2 mRNA in knockdown FOXO3a in CL1-5/ANGPTL1.

Fig. 5a-f

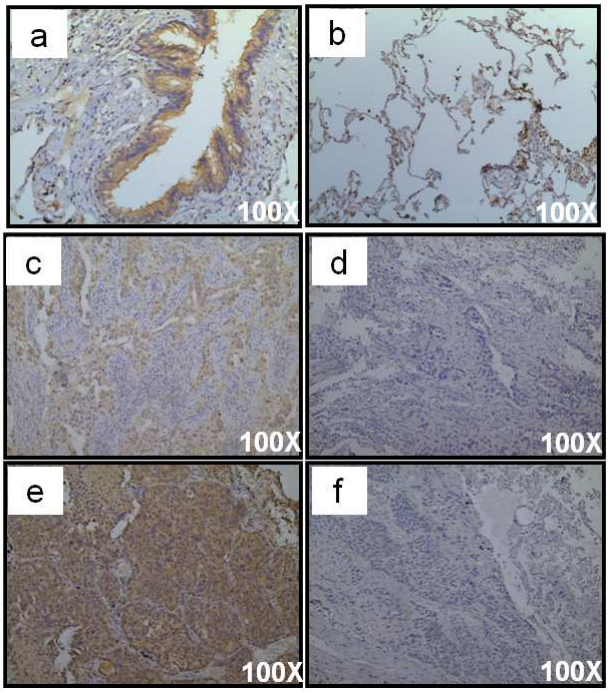

Fig. 5g

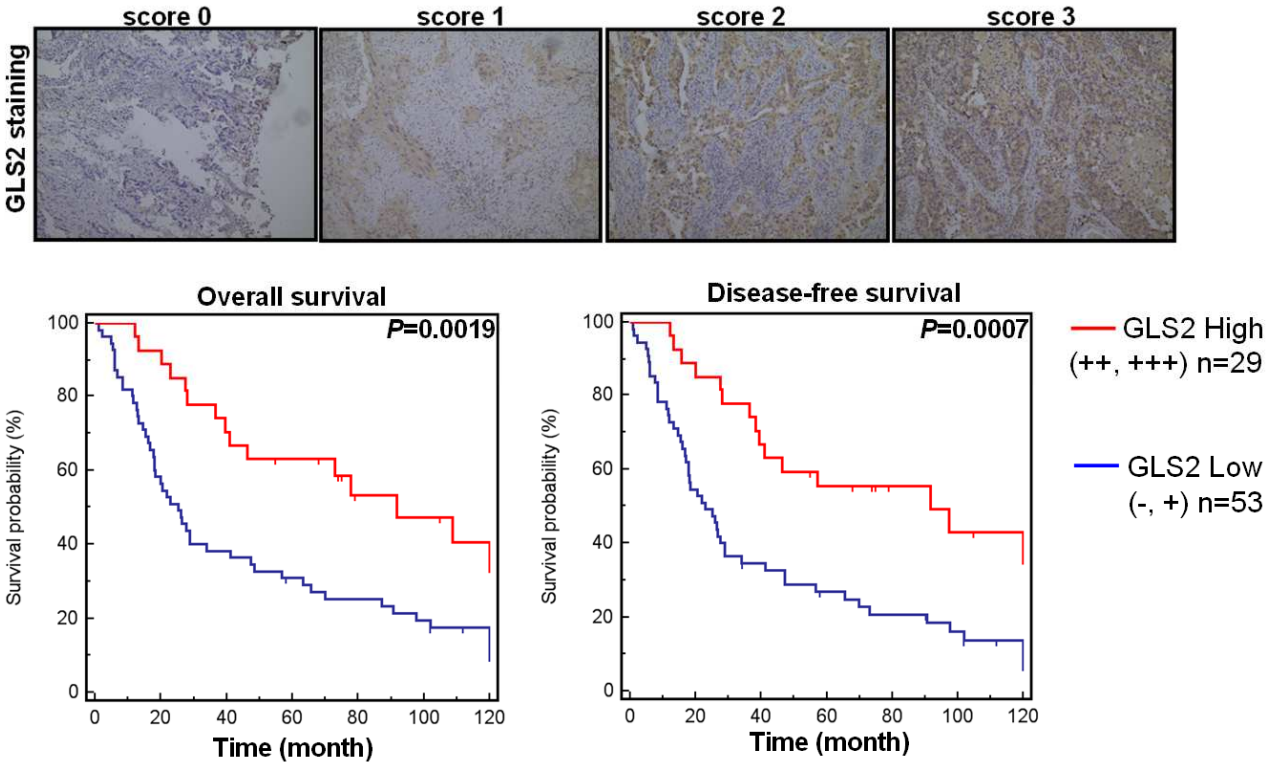

Fig. 5h

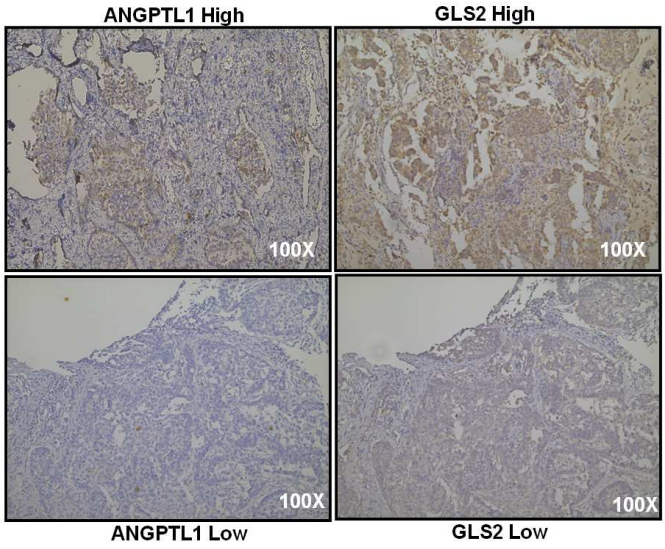

Fig. 5. GLS2 is expressed in cancers and inversely correlates with survival of cancer patients. (a–f) GLS2 levels in representative normal and tumor tissues. Immunohistochemistry using the rabbit anti-GLS2 antibody (ABGENT). (a) (b) Normal lung tissue. (c)(d) Lung adenocarcinoma tissues. (e)(f) Lung squamous cell carcinoma tissues. (c) and (e) are examples of high GLS2 expression; (d) and (f) are examples of low GLS2 expression. (g) Kaplan-Meier plot of overall and disease-free survival of 82 patients with lung carcinomas stratified by GLS2 expression level. A log rank test was used to show differences between groups.

Table

Table. 1 Univariants and multivariate analysis of potential prognostic variables

Table 1. Univariants and multivariate analysis of potential prognostic variables

Parameters	Comparison	Univariate analysis		Multivariate analysis	
		HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
ANGPTL1 score	Low (0, 1); High (2, 3)	0.5515 (0.3195 to 0.9518)	0.03346*	0.6583 (0.3699 to 1.1715)	0.1572
GLS2 score	Low (0, 1); High (2, 3)	0.4119 (0.2309 to 0.7349)	0.002812*	0.3262 (0.1737 to 0.6128)	0.0005301*
Pathological stage	I-II ; III-IV	2.119 (1.916 to 3.397)	0.009252*	2.4770 (1.1876 to 5.1662)	0.01612*
Tumor status	T1-T2; T3-T4	1.2415 (0.7462 to 2.0657)	0.4073	0.8667 (0.5055 to 1.4860)	0.6049
Lymph nodal status	N0;N1-N3	2.0451 (1.2429 to 3.3650)	0.005089*	1.5991 (0.8382 to 3.0509)	0.1564
Metastasis	Yes; No	1.0851 (0.4363 to 2.6989)	0.8612	0.6098 (0.2095 to 1.7748)	0.3666

NOTE: Cox proportional hazards regression was used to perform uni- and multivariant analysis for potentially important variates.

Abbreviations: HR, hazard ratio; CI, confidence interval.

***Two-sided Cox proportional hazards regression using normal approximation.**

Table. 2 correlation between level of ANGPTL1 and GLS2 expression in lung cancer

Table 2 correlation between level of ANGPTL1 and GLS2 expression in lung cancer

	ANGPTL1[a]			
GLS2[a]	-	+	++	+++[b]
-[b]	23	5	2	5
+	7	4	5	3
++	7	4	6	1
+++	3	0	4	3

a: Detection of the expression of ANGPTL1 and GLS2 immunohitochemistry
b: Intensity of immunohistochemical staining: -, negative; +,0-10% tumor cells stained; ++, 10%-50% of tumor cells stained; +++, >50% tumor cells stained.