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Signaling Pathways and Inhibitors of Cells from Patients with Kaposiform Lymphangiomatosis

Elisa Boscolo^{1,2}, Patricia Pastura^{3,2}, Kathryn Glaser², Jillian Goines^{1,2}, Adrienne M. Hammill^{2,4}, Denise M. Adams^{5,6,8}, Peter Dickie², Belinda Hsi Dickie^{6,7,8}, and Timothy D. Le Cras^{2,3}

¹Division of Experimental Hematology and Cancer Biology, University of Cincinnati College of Medicine, Cincinnati, OH, USA

²Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, OH, USA

³Division of Pulmonary Biology, Cincinnati Children's Hospital, Cincinnati, OH, USA

⁴Hemangioma & Vascular Anomalies Center & Division of Hematology, Cincinnati Children's Hospital, Cincinnati, OH, USA

⁵Vascular Anomalies Center, Boston, MA, USA

⁶Boston Children's Hospital, Boston, MA, USA

⁷Colorectal and Pelvic Malformation Center, Boston, MA, USA

⁸Harvard Medical School, Boston, MA, USA

Abstract

Background.—Kaposiform lymphangiomatosis (KLA) is a rare lymphatic anomaly with significant morbidity and mortality. KLA is characterized by diffuse multifocal lesions comprised of focal areas of “kaposiform” spindled cells accompanying malformed lymphatic channels. The goal of this study was to identify activated signaling pathways in cells isolated from three KLA patients for the purpose of testing new therapies.

Procedure.—Cells were obtained from the lungs of one patient isolated at autopsy and the spleen of two patients removed in surgery due to disease complications. A protein kinase array was performed on the KLA cell lysates and normal lymphatic endothelial cells.

Results.—Higher activation of key signaling pathways in the KLA cells, including PRAS40, AKT1/2/3 and ERK-1/2, was identified by protein kinase array and confirmed by western blot analysis. This indicated a role for highly activated PI3 Kinase (PI3K) - AKT and mitogen activated protein kinase (MAPK)/ERK-1/2 signaling pathways in KLA cells. Cell proliferation studies assessed PI3K inhibitors (LY294002; BYL719), AKT inhibitor ARQ092, mTOR inhibitor

Correspondence to: Tim Le Cras, Ph.D., Division of Pulmonary Biology, Cincinnati Children's Hospital Medical Center, 3333 Burnet Avenue, Cincinnati, Ohio 45229-3039; tim.lecras@cchmc.org; Phone: 513-636-8151; Fax: 513-636-7868.

CONFLICT OF INTEREST STATEMENT

The authors have nothing to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

rapamycin and MAPK inhibitor U0126. These studies demonstrated that PI3K-AKT-mTOR and MAPK signaling are important mediators of KLA cell proliferation. BYL719 and rapamycin were more effective at inhibiting KLA cell proliferation than U0126.

Conclusions.—Our studies using cells from KLA patient lesions demonstrate that these cells are highly proliferative and the PI3K-AKT-mTOR and MAPK pathways are promising therapeutic targets. Development and clinical trials of PI3K, AKT, and MAPK inhibitors for cancer treatment and the data in this study lend support for early clinical trials assessing the efficacy of these inhibitors in KLA patients.

Keywords

Vascular Malformations; All, molecular diagnosis & therapy; Pharmacology

INTRODUCTION

Lymphatic malformations (LM) are vascular anomalies that affect infants and children of any age and gender. Kaposiform lymphangiomatosis (KLA) is a recently identified¹ rare LM that may be a subtype and/or more severe form of generalized lymphatic anomaly (GLA). The pathology of KLA is characterized by multifocal areas of spindle-shaped “endothelial-like” cells in sheets/clusters that are associated with abnormal diffuse lymphatic channels¹. Patients with KLA suffer from high morbidity and mortality with 51% survival after 5 years² and overall survival rate of 34%¹. This poor prognosis is due to a constellation of life-threatening symptoms including pleural and pericardial effusions and ascites, consumptive coagulopathy, and hemorrhage^{1,3}. Pulmonary and abdominal involvement is especially associated with poor outcome. Diagnosis and therefore treatment of KLA is often delayed as the disease is rare, usually misdiagnosed, and thus needs highly specialized expertise in imaging and pathology, with biopsy carrying considerable risk for bleeding. A number of different treatments have been used in KLA patients, although the most widely used to date is the mTOR inhibitor sirolimus (rapamycin)^{2,4}. Unfortunately not every KLA patient responds⁵ to sirolimus and some experience rebound disease if therapy is discontinued. Hence there is a significant need for additional therapeutic options which will require a better understanding of the etiology of KLA.

The aim of this study was to determine key activated signaling pathways in cells from KLA patients and so aid identification of new therapeutic targets. Cells isolated from the lung and spleens of KLA patients were utilized⁶. The ability to study these cells, even though they only represent three patients, is a valuable opportunity as KLA is rare and cells have only been isolated from a few patients since this disease was first identified. A protein kinase array of KLA cell lysates was performed and this identified a number of activated kinases and related pathways including phosphoinositide 3-kinase (PI3K) and mitogen activated protein kinase (MAPK). The role of these pathways in KLA cell proliferation was tested using inhibitors of PI3K/AKT, mTOR and MAPK/ERK pathways. We used some standard inhibitors of PI3K (LY290042), mTOR (rapamycin) and ERK (U0126), as well as a new PI3K α -selective inhibitor BYL719 (Alpelisib)⁷ and a pan-AKT (protein kinase B) inhibitor ARQ092 (Miransertib)⁸. BYL719 and ARQ092, have shown some early promise in pre-clinical studies and early phase of clinical trials^{7,9–11}.

METHODS

Patient Characteristics

All diagnoses were confirmed by radiologic and histopathologic examination. Table 1 lists information on the KLA patients. Patient samples were obtained with written informed consent in accordance with Institutional Review Board guidelines and approval. De-identified tissue was obtained from patients undergoing surgical procedures or postmortem. Tissues from external centers was shipped on ice to Cincinnati Children's Hospital Medical Center by overnight carrier and then processed for cell isolation immediately upon arrival.

KLA Cell Isolation and Characterization

For cell isolation minced tissue was homogenized enzymatically with 0.2% collagenase type I (Worthington Biochemical Corp.) in Dulbecco Modified Eagle Medium and passed through a 100 μ M nylon sieve⁶. Cells were collected by centrifugation and expanded on fibronectin-coated plastic. Normal human neonatal dermal lymphatic endothelial cells (LEC) were obtained from Lonza (catalog number CC-2812). LEC and KLA cells were grown in endothelial basal medium (EBMTM-2) (Lonza; catalog number CC-3156) plus endothelial growth factors kit (Lonza; EGMTM-2 MV SingleQuotsTM supplements CC-4147) and fetal bovine serum (FBS) at 20% (v/v). This media is hereafter referred to as endothelial growth medium (EGM-2MV). Cells were used between passages 7–20.

Protein Kinase Array and Western Blot Analysis

Protein kinase activity was analyzed using a human phospho-kinase array (R&D systems; catalog number ARY003B) to identify signaling pathways that were especially active in the KLA cells compared to LEC. Cell lysates (375 μ g protein) were prepared from confluent cells cultured in EGM-2MV media. Array assays were performed according to manufacturer's protocol and imaged using Kodak X-ray film. Films were imaged on a scanner and signals quantitated by densitometry. Results from the array were confirmed by performing western blot analysis on cell lysates with antibodies for phospho-AKT (Cell Signaling Technology; Ser473 catalog number 4060 and Thr308 catalog number 2965), phospho-Proline-Rich AKT Substrate of 40 kDa (Cell Signaling Technology; p-PRAS40 Thr246; catalog number 22997), phospho-p44/42 MAPK (ERK1/2)(Cell Signaling Technology; catalog number 9101). Total AKT (Cell Signaling Technology; catalog number 9272), PRAS40 (Cell Signaling Technology; catalog number 2691), ERK-1/2 (Cell Signaling Technology; Cell Signaling Technology; catalog number 9102), and C4 actin (Chemicon; catalog number MAB1501) were also assessed.

Cell proliferation and PI3K/AKT/mTOR and MAPK/ERK Inhibitors

Proliferation studies were conducted comparing KLA cells to LEC. Cells were plated in 96 well plates (6×10^5 cells per well) coated with fibronectin in EGM-2MV media. Medium was replaced 12 hours after plating the cells and this was designated time zero (T=0). Proliferation was measured using the Sulforhodamine B (SRB) method¹² at 0, 24, 48, and 72 hours. For the inhibitor studies cells were plated in 96 well plates (8×10^5 cells per well) coated in fibronectin in EGM-2MV media. The media was replaced 5 hours after plating

(Time 0) with EGM-2MV media containing inhibitors or the vehicle control (dimethyl sulfoxide; DMSO). Inhibitors to PI3K (LY294002 10 μ M; BYL719 10 μ M), AKT/protein kinase B (ARQ092 5 & 10 μ M), MAPK (U0126 10 μ M), rapamycin (sirolimus)(15nM) dissolved in DMSO were used. Inhibitors were purchased from Selleckchem (LY294002 catalog number 15447–36-6; BYL719 catalog number 1217486–61-7; ARQ092 catalog number 1313883–00-9; U0126 catalog number 1173097–76-1) or LC Laboratories (Rapamycin; catalog number R-5000). DMSO was added to control cell wells to match the volume used for inhibitors (0.2% vol/vol). Cell numbers were measured 72 hours after plating using the SRB method¹². Western blot analysis was also performed on cell lysates 1 hour after treatment with inhibitors to assess inhibitory effects on p-AKT and p-ERK1/2.

DNA sequencing.

The presence of common *PIK3CA* mutations in the KLA cells was assessed by DNA Sanger sequencing as previously described¹³. Briefly, amplified DNA product was generated by PCR using primers for *PIK3CA* exons 7, 9, and 20 (Integrated DNA Technologies). PCR products were sequenced at the CCHMC DNA Sequencing and Genotyping Core. The *NRAS* gene was analyzed for mutations using molecular inversion probe sequencing¹⁴.

Data and Statistical Analysis

Graphing and statistical analysis were performed using Prism 7 software. Cell proliferation time course data was converted to show fold changes from baseline and then analyzed in log-scale to counter for skewness. Two-way Analysis of Variance was performed with cell group and time point as factors, along with their interaction. For the inhibitor studies, mean percent inhibition of cell proliferation was calculated relative to vehicle control. Confidence intervals (95%) for the means were calculated to show the magnitude and certainty of the inhibition. One-way ANOVA and Tukey's multiple comparison method was used to compare the effects of the different pharmacologic inhibitors on KLA cell proliferation.

RESULTS

Patient Characteristics and Cell Isolation

Table 1 shows summary data for 3 KLA patients seen at different US Children's Hospitals. All patients had been treated with prednisone and sirolimus. In addition, patient 1607 had also been treated with vincristine. Cells were isolated at post-mortem from the lungs of a 14-year-old female patient (1607) with KLA involvement of multiple organs. Cells were derived from the spleens of a 5-year-old male (1528) and 10 month-old female (2360) at surgery.

KLA Cell Characteristics

Cells from the three KLA patients are shown at near confluence along with normal lymphatic endothelial cells (LEC)(Fig. 1). Immunostaining of fixed KLA cells and LEC for D2–40 (podoplanin) is shown in Fig. 1. KLA cells were spindle-shaped under phase contrast and stained positive for D2–40 (podoplanin), as previously reported⁶.

Protein Kinase Array and Western Blot Verification

Supplemental Fig. 1A shows a representative kinase array blot using cell lysates from LEC and KLA 1607 cells (unstimulated). Examples of protein targets that were elevated in KLA patient cells compared to LEC are shown. After densitometry, histograms of the data (Supplemental Fig. 1B) showed higher levels of phosphorylated proteins in KLA cells relative to LEC that included PRAS40 (Thr246), AKT1/2/3 (Ser473) and ERK1/2 (Thr202/Tyr182, Thr185/Tyr187). For p-HSP60 only one KLA cell type (1607) showed elevated levels and so this was not pursued further. Western blot analysis was used to confirm that activation levels of PRAS40, AKT and ERK1/2 were higher in KLA cells than LEC (Fig. 2). Greater signal for p-AKT (Ser473 and Thr308), p-PRAS40 (Thr246), p-ERK1/2 were confirmed in all 3 KLA cells. Total PRAS40 levels also were higher in KLA cells. While p-AKT Thr308 was not elevated in the kinase array, on western blot analysis it was higher than LEC although more variable between the KLA patients than p-AKT Ser473.

Cell Proliferation and Signaling Pathway Inhibitors

Cell proliferation studies were used to assess the proliferation rates of the KLA cells in 20% FBS (Fig. 3). The KLA cell group had a significantly higher proliferation rate than LEC cells ($p=0.0003$). There was also a significant increase in proliferation with time ($p=0.0063$). Next, we assessed the effects of PI3K, AKT, mTOR and MAPK inhibitors on the activated pathways and cell proliferation. Fig. 4A shows western blot analysis 1 hour after treatment to assess the inhibition of p-AKT and p-ERK levels by the inhibitors. The PI3K/AKT inhibitors LY290042, BYL719, ARQ092 ablated AKT phosphorylation while U0126 decreased ERK-1/2 activation in all the KLA cells analyzed and LEC. Furthermore, LY290042, BYL719, ARQ092, rapamycin and the MAPK inhibitor U0126 prevented KLA and LEC cell proliferation (Fig. 4B). Mean inhibition (percentage) and confidence intervals indicate that there was significant inhibition of proliferation with the inhibitors used to treat the KLA cells (Table 2). In our preliminary studies 10 μ M ARQ092 completely blocked cell proliferation and so 5 μ M was used for all studies reported here. By Tukey's multiple comparison method, we found that inhibition of proliferation of KLA cells with BYL719 and rapamycin treatments were significantly different from U0126 ($p=0.0163$ and $p=0.0289$, respectively). There was no evidence of significance among the other groups. In summary, all the inhibitors tested efficiently inhibited proliferation of KLA cells, with BYL719 and rapamycin more effective than U0126.

Mutation Analysis

No hotspot mutations in PIK3CA (C420 mutation in exon 7, E542, E545, E546 mutations in exon 9, and H1047 mutations in exon 20) were found in the KLA cells. All samples were sequenced to a depth across the entire NRAS coding sequence of >1000X and no missense mutations affecting residues p.G12, p.G13, or p.Q61 were observed at greater than the background rate for this technique.

DISCUSSION

The cellular basis for KLA are not well understood although both abnormal LEC channels and "spindle cells" that express LEC markers are characteristic of the histopathology of

patient lesions. In a study by Glaser et al⁶, cells derived from KLA patients were found to be highly proliferative compared to adipose-derived mesenchymal stem cells (ADSCs). In our study we utilized KLA cells from two of the patients in the Glaser study⁶, (KLA 1 and KLA2) (1528 cells from spleen and for patient 1607 cells were from the lung), as well as cells derived from the spleen of an additional KLA patient (2360). We compared KLA cells to LEC since they have some similarities to the spindle cells in KLA lesions. While the choice of a normal cell type to compare the KLA cells to is a limitation, the main goal of our study was to identify key signaling pathways mediating proliferation of cells from KLA patient lesions to identify potential new therapeutic targets. Our kinase array study and verification with western blot analysis showed high AKT activation as well as MAPK. Hyperactive PI3K-AKT-mTOR signaling has also been found in a number of vascular malformations^{14–19}.

We did not find any of the common PIK3CA mutations in the KLA cells that have been found in vascular anomalies associated with tissue overgrowth including Klippel-Trenaunay syndrome (KTS) and congenital lipomatous overgrowth vascular malformations epidermal nevi and scoliosis/skeletal/spinal anomalies (CLOVES)^{15–19}. In the Glaser study⁶, whole exome sequencing of KLA-derived cells identified a potential pathogenic variant in one KLA patient (which is patient 1607 in our study), a 3-base pair deletion resulting in loss of Serine 1043 in tuberous sclerosis complex 1 (TSC1). TSC1 is an inhibitor of mTOR an important signaling mediator downstream of PI3K/AKT a major growth/survival pathway in angiogenesis. While the exact significance of the deletion in TSC1 that Glaser et al⁶ found is unclear, our studies indicate that the PI3K/AKT/mTOR pathway is very active in KLA cells. We also found high phosphorylation of PRAS40 in KLA cells. PRAS40 is downstream target of AKT, a component of the mTOR complex 1 (mTORC1) that regulates mTOR activity, and when phosphorylated dissociates from mTORC1 so that it can function²⁰. PRAS40 is increasingly used as a biomarker of AKT activation in tumors²¹. The mTOR inhibitor sirolimus (rapamycin) has been used to treat KLA patients with a partial response in KLA patients². Of the KLA patients who responded to therapy, all continued sirolimus treatment without any disease progression although long-term efficacy in these patients is still unclear. While sirolimus is now commonly used to treat KLA patients, disease symptoms can rebound if patients discontinue treatment. Recently Barclay et al²² identified an *NRAS* activating mutation p.Q61R in tissue from KLA patients. This *NRAS* mutation can increase MAPK and PI3K signaling. While the *NRAS* variant was present in the tissue of the majority of patients analyzed in Barclay's study²², the allelic frequency ranged from 1% to 28% and so not all cells in the lesions had the mutation. It remains unclear what cell type(s) in the KLA lesions harbor the *NRAS* mutation and also which cell type actually drives the disease process although spindle cells and abnormal LEC are potential contributors^{1,3}. We did not find the *NRAS* gene mutation (p.Q61R) in the cells from the KLA patients in our study. However, the PI3K and MAPK signaling pathways were contributing to cell proliferation suggesting a common mechanism.

A number of PI3K inhibitors have been developed and are in clinical trials for cancer therapeutics²³. These include pan-PI3K and PI3K isoform-selective inhibitors; PI3K inhibitor that is selective for the δ isoform, Idelalisib, is now approved for treatment of chronic lymphocytic leukemia, relapsed follicular B-cell non-Hodgkin's lymphoma and

relapsed small lymphocytic lymphoma. BYL719 (Alpelisib) is a specific inhibitor of the PI3K isoform α (encoded by PIK3CA gene) that has shown some early promise in PI3K-altered solid tumors⁷ and in patients with CLOVES¹¹. ARQ092 (Miransertib) is a new allosteric inhibitor of AKT that is orally bioavailable and has a manageable safety profile in patients with advanced solid tumors. Studies using fibroblasts from PROS patients showed that ARQ092 had good antiproliferative activity²⁴ and has now been granted “Fast Track” designation by the Food and Drug Administration for treatment of PROS as well as Rare Pediatric Disease designation for treatment of Proteus syndrome²⁵. Based on this promising background we thought that it might be beneficial to evaluate this new drug in cells from KLA patients to generate data of pre-clinical interest since these cells have hyperactive AKT. We found that both BLY719 and ARQ092 inhibitors were effective at inhibiting growth of KLA cells at similar doses to LY294002, that is an older generation PI3K inhibitor. BYL719 and rapamycin were also significantly better at inhibiting KLA cell proliferation than the MAPK inhibitor U0126.

In conclusion, our studies using cells from KLA patient lesions demonstrate that they are highly proliferative and that inhibition of PI3K and AKT may represent potential therapeutic targets. Novel findings are the identification of the signaling pathways that mediate KLA cell proliferation and the use of inhibitors that are in clinical trials for other cancer therapy, as well as vascular anomalies and overgrowth syndromes. The data from this study may lend support for the pre-clinical testing of these novel PI3K and AKT inhibitors in KLA patients. Future studies may also need to include dual inhibitor agents if the toxicity profile is adequate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS KEY

KLA	Kaposiform lymphangiomatosis
PI3K	phosphoinositide 3-kinase
MAPK	mitogen activated protein kinase
GLA	generalized lymphatic anomaly
FBS	fetal bovine serum

EGM-2MV	endothelial growth medium
SRB	Sulforhodamine B
DMSO	dimethyl sulfoxide
α-SMA	α -smooth muscle actin
ADSCs	adipose-derived mesenchymal stem cells
KTS	Klippel-Trenaunay syndrome
CLOVES	congenital lipomatous overgrowth, vascular malformations, epidermal nevi and scoliosis/skeletal/spinal anomalies
TSC1	tuberous sclerosis complex 1
PRAS40	phospho-Proline-Rich AKT Substrate of 40 kDa
mTORC1	mTOR complex 1

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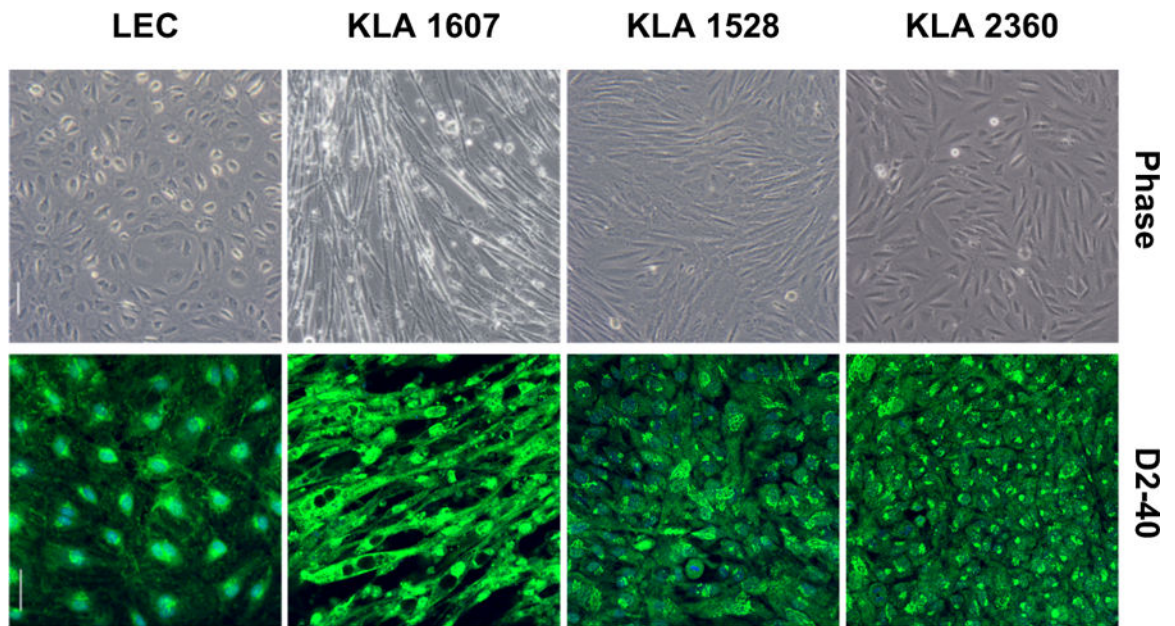


Fig. 1. Cells from KLA patients and neonatal human lymphatic endothelial cells (LEC) cultured on fibronectin-coated plates. Phase contrast pictures were taken using a 10X objective, scale bar is 200 μ M. Pictures of immunostaining for D2-40 (podoplanin) immunofluorescence pictures were taken using a 40X objective, scale bar is 50 μ M.

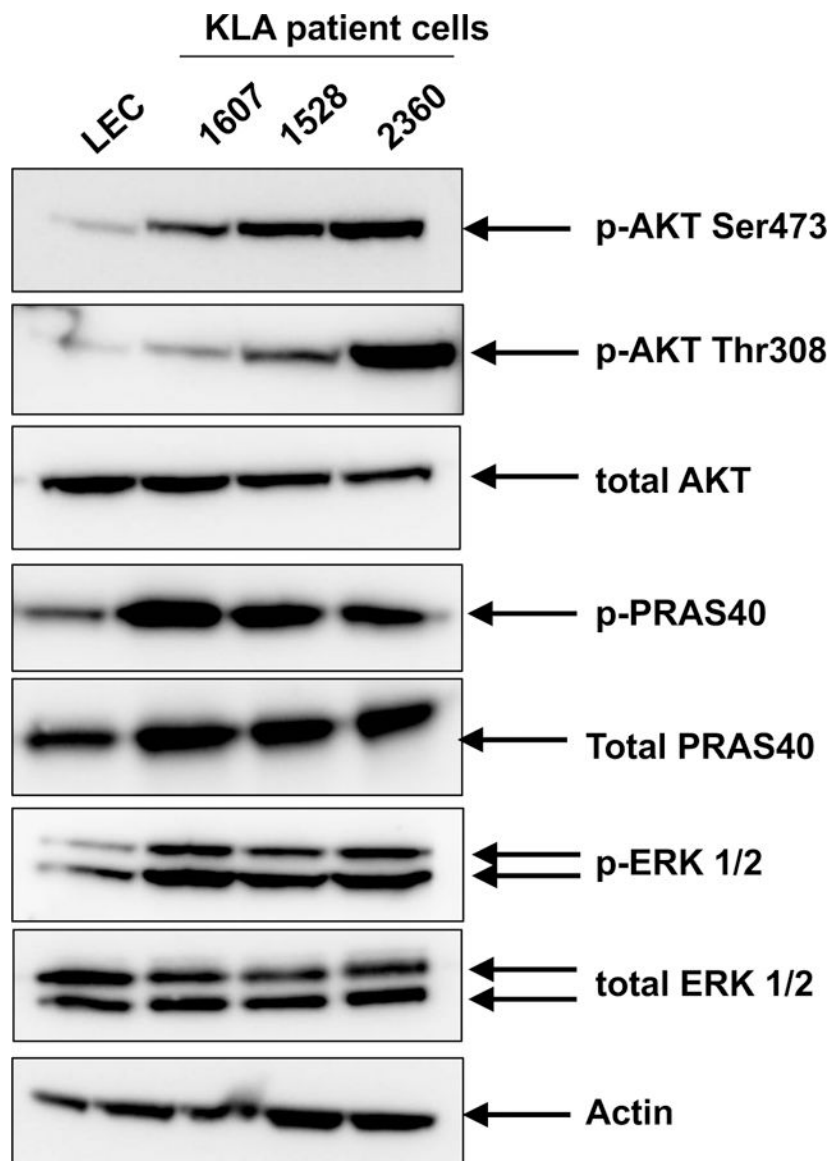


Fig. 2. AKT and ERK phosphorylation in cells from KLA patients and neonatal human lymphatic endothelial cells (LEC). Western blot analysis was performed on cell lysates (20 μ g total protein) from KLA cells and LEC. Blots were probed for phosphorylated-AKT (p-Ser473 and p-Thr308), total AKT, p-PRAS40 (downstream target of AKT), total PRAS40, p-ERK1/2, total ERK1/2 and actin.

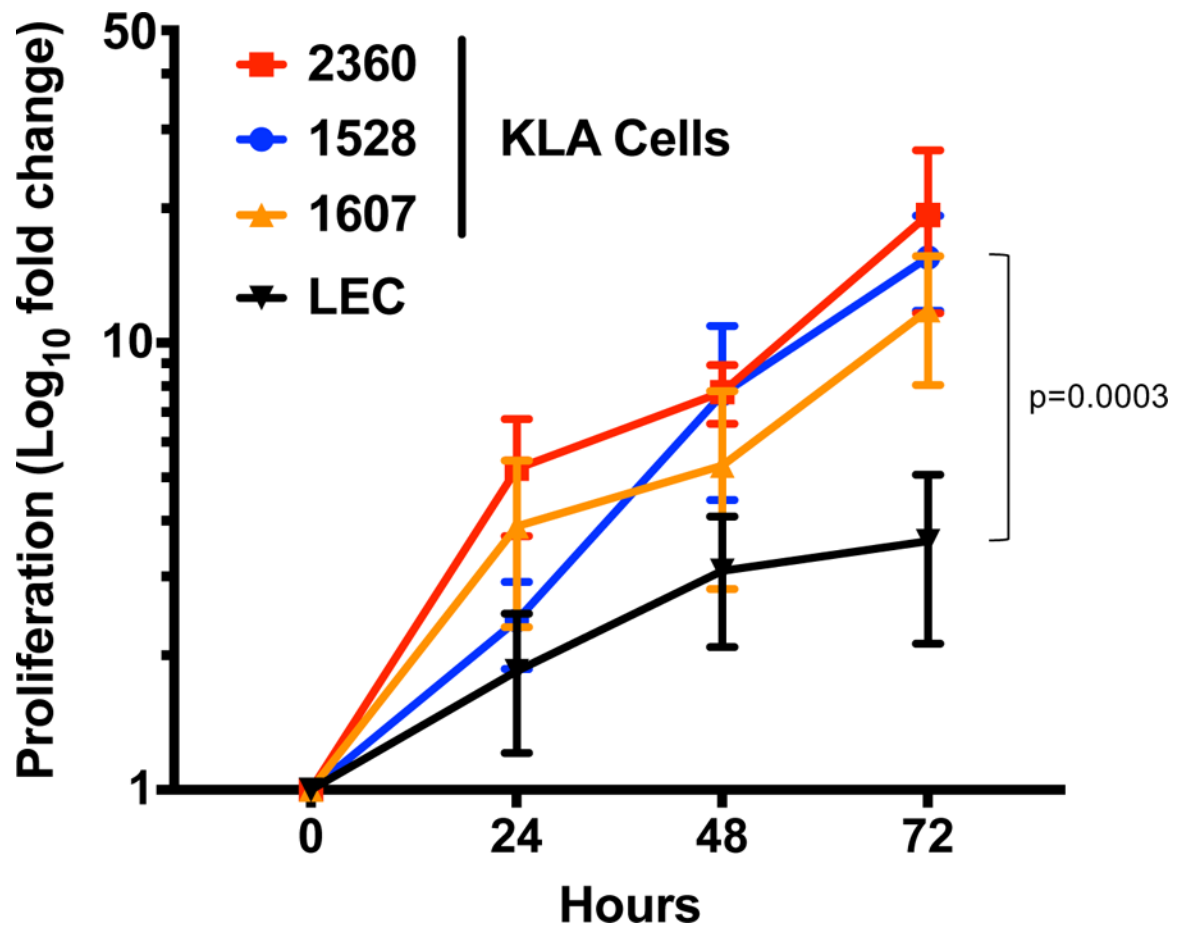


Fig. 3. Proliferation of cells from KLA patients and human lymphatic endothelial cells (LEC). Cells were cultured in 20% FBS in EGM-2MV media on fibronectin-coated 96 well plates. Cell number was assessed at 0, 24, 48 and 72 hours using SRB assay. Cell numbers are expressed as Log₁₀ of fold change in cell number compared to time 0. Data represents the results from 3 independent experiments.

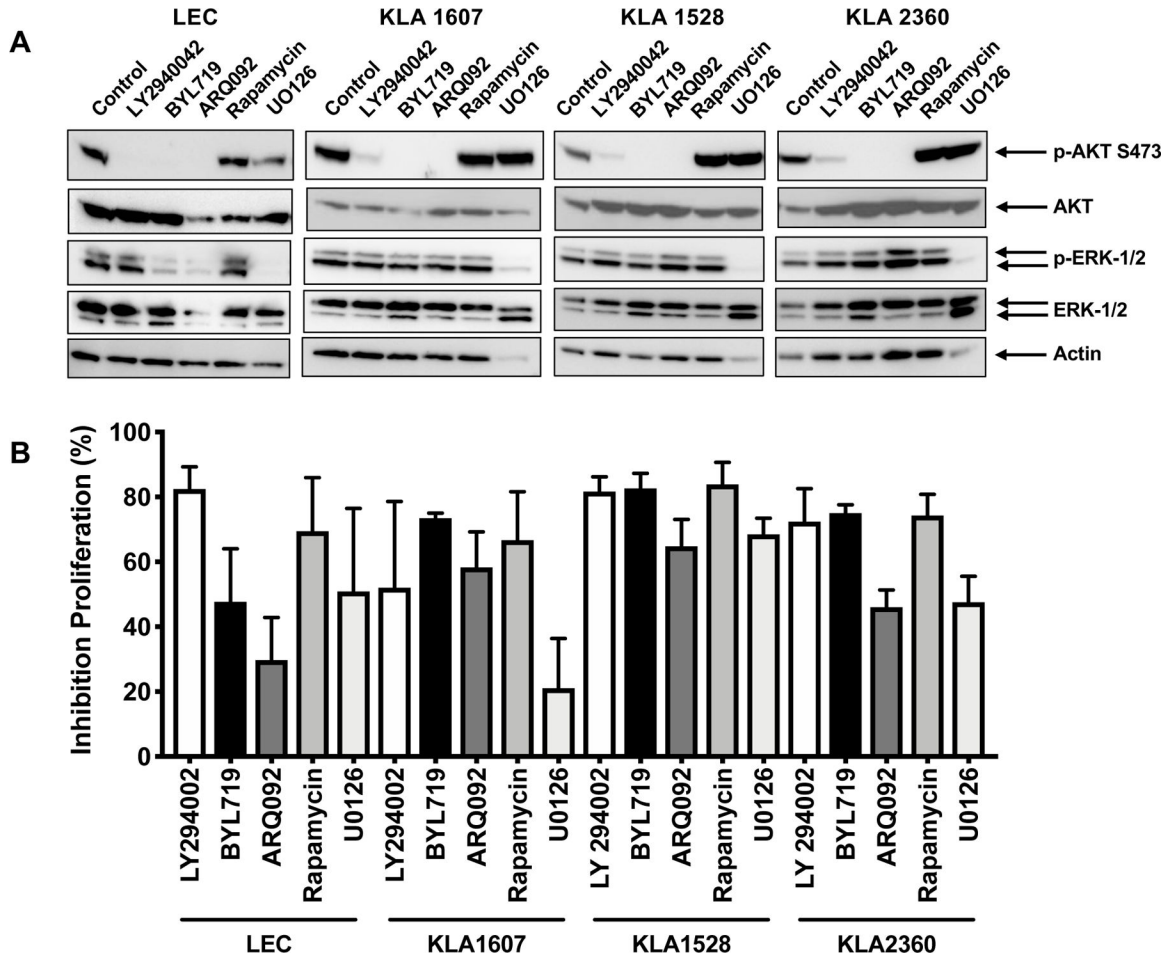


Fig. 4.
A. Inhibition of AKT and ERK1/2 phosphorylation in KLA cells and human lymphatic endothelial cells (LEC) treated with inhibitors. Western blot analysis was performed on cell lysates (10µg total protein) from KLA cells (1607, 1528, 2360) and LEC, after treatment (1 hour) with either DMSO vehicle (control), PI3K inhibitors LY294002 (10µM) and BYL719 (10µM), AKT inhibitor ARQ092 (5µM), rapamycin (15nM) or MAPK inhibitor U0126 (10µM). Blots were probed with antibodies to phosphorylated-AKT (pSer473), total AKT, p-ERK1/2, total ERK and actin. **B.** KLA cells and LEC were plated on fibronectin-coated plates in EGM-2MV media. After plating (5 hours) the media was changed to media containing either DMSO vehicle (control), PI3K inhibitors LY294002 (10µM) and BYL719 (5µM), AKT inhibitor ARQ092 (5µM), rapamycin (15nM) or MAPK inhibitor U0126 (10µM). Proliferation was measured using SRB assay 72 hours after treatment with inhibitors. Data represents the results from 3 independent experiments and show percent inhibition compared to vehicle control.

TABLE 1

KLA Patient Information

Patient Number	Tissue Used to Isolate Cells	Center	Age	Sex
1607	Lung	Texas Children's	14 years	Female
1528	Spleen	Cincinnati Children's	5 years	Male
2360	Spleen	UCSF*	10 months	Female

* University of California San Francisco

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TABLE 2

Inhibition of Cell Proliferation

KLA Cells	LY294002 10 μ M	BYL719 10 μ M	ARQ092 5 μ M	Rapamycin 15nM	U0126 10 μ M
Mean Inhibition (%)	69	77*	56	75**	46
Std. Deviation	28	6	15	17	26
95% CI of mean	47–90	72–82	45–68	62–88	26–66

* BYL719 is significantly different from U0126 $p=0.0163$ (Tukey's method)

** Rapamycin is significantly different from U0126 $p=0.0289$ (Tukey's method)

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