Published in final edited form as:

Peptides. 2010 August ; 31(8): 1511–1516. doi:10.1016/j.peptides.2010.04.017.

Protein expression of urotensin II, urotensin-related peptide and their receptor in the lungs of patients with lymphangioleiomyomatosis

Arnold S. Kristof, Zhipeng You, Yin-Shan Han, and Adel Giaid*

Department of Medicine, McGill University Health Center, Montreal, Quebec, Canada.

Abstract

Urotensin II (UII) and urotensin-related peptide (URP) are vasoactive neuropeptides with wide ranges of action in the normal mammalian lung, including the control of smooth muscle cell proliferation. UII and URP exert their actions by binding to the G-protein coupled receptor-14 known as UT. Lymphangioleiomyomatosis (LAM) is a disease of progressive lung destruction resulting from the excessive growth of abnormal smooth muscle-like cells that exhibit markers of neural crest origin. LAM cells also exhibit inactivation of the tumor suppressor tuberin (TSC2), excessive activity of 'mammalian target of rapamycin (mTOR), and dysregulated cell growth and proliferation. In the present study we examined the expression and distribution of U-II and UT in the lungs of patients with LAM. There was abundant expression of UII, URP and UT proteins in the interstitial nodular lesions of patients with LAM. By immunohistochemistry, UII, URP and UT were co-localized with HMB45, a diagnostic marker of LAM. Immunoreactivity for UII, URP and UT was also evident over the pulmonary epithelium, pulmonary vasculature and inflammatory cells. Western blotting revealed the presence of greater UT expression in the lungs of patients with LAM compared to normal human lungs. UT expression correlated with mTOR activity, as indicated by increased phosphorylation of S6 in LAM samples. These findings demonstrate for the first time the presence of UII, URP and their receptor in the lesions of patients with LAM, and suggest a possible role in the pathogenesis of the disease.

1. Introduction

Urotensin II (UII) is a small ancient peptide (11–14 amino acids) that was originally isolated from the urophysis, a neurosecretory organ, of the goby fish [3,43]. The peptide is conserved from invertebrates to humans, shares similar structure to somatostatin, and is expressed in the nervous system and cardiopulmonary system of various species [1]. UII has a wide range of physiologic actions mediated through binding to the G-protein coupled receptor-14 named UT [1,33,40,42]. Pharmacologic studies have shown UII to be the most potent vasoconstrictor peptide identified to date, though its vasoactive properties vary depending on the species and type of vessels investigated [14].

© 2010 Elsevier Inc. All rights reserved.

*Address for correspondence: Dr. Adel Giaid, Cardiology, The Montreal General Hospital, 1650 Cedar Avenue, Suite L3-109, Montreal, Quebec H3G 1C6, Canada, Tel: 514 934 1934 ext: 43841, Fax: 514 934 8344, adel.giaid@mcgill.ca.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

The bioactivity of UII is highly variable, and has been attributed in part to the levels of UT in any given tissue [15]. For example, UII constricted human coronary, radial and pulmonary arteries, which are tissues that exhibit abundant expression of UT [1,17,18,36]. On the other hand, UII can also exert endothelium-dependant vasorelaxation through release of nitric oxide and prostaglandin I₂ in the aorta, as well as coronary and mesenteric arteries [4,22,23,31,32, 50]. Others reported no significant effect for UII on pulmonary arteries of humans and animals [50]. Apart from its vascular effects, UII contracted airway smooth muscle cells of human, cat and rat bronchi [2,24]. UII also stimulates proliferation of the smooth muscle cells of pulmonary and systemic arteries [12,46,56], epithelial cells [39], cardiac [25] and adventitial fibroblast [10], as well as umbilical endothelial cells [48,49].

UII is expressed mainly in the aorta, heart, kidneys, central nervous system and spinal cord [1]. The UII receptor, UT, is expressed in the heart, brain, lungs, brain, skeletal muscles, bladder and pancreas [1,28,33,40,42]. UT is also expressed in vascular and airway smooth muscle cells, as well as pulmonary vasculature [37,38]. Recent studies have demonstrated the presence of another ligand for UT named urotensin-related peptide URP [9,51]; the detailed expression of URP in the human lungs remains to be elucidated. The proliferative effects of UII, a neuropeptide, suggested a potential role in neoplastic diseases of neuroendocrine origin. In agreement, increased UT expression, as well as proliferation in response to UII, was demonstrated in adrenal tumors [53,57]. We hypothesized that UII and its receptor might play a role in lymphangioleiomyomatosis (LAM), a pulmonary and lymphatic disease caused by abnormal smooth muscle-like cells that also express neural crest markers.

Lymphangioleiomyomatosis (LAM) is a rare disease of young women characterized by abnormal proliferation of smooth muscle-like cells (LAM cells) in the pulmonary interstitium. The nodular proliferation of LAM cells leads to cystic destruction of the lungs and respiratory failure. Recent evidence describing the recurrence of LAM after lung transplantation and the presence of LAM cells with the same mutation in the lung, lymph nodes and kidney suggests that LAM cells are clonal, and can metastasize [29]. Although the origin of LAM cells is unknown, they express markers of smooth muscle differentiation (e.g., α -actin), as well as proteins associated with neural crest lineage (e.g., GP100). Recognition of GP100, by the HMB45 antibody is diagnostic of LAM. Given that urotensin signaling might play an important role in the pathophysiology of neoplastic processes of neural lineage, we sought to determine the expression of U-II, URP and UT in the lungs of patients with LAM and compare that to those of normal individuals.

2. Materials and Methods

2.1. Tissues

Lung tissues from 7 patients with LAM (age 45 $\pm$ 2 years) and 6 normal control subjects (age 42 $\pm$ 5 years) collected either at transplantation or at autopsy were either snap frozen in liquid nitrogen or fixed in 10% formalin. Anonymized frozen or formalin-fixed lung specimens from patients with LAM were obtained from the National Disease Research Interchange or the NHLBI LAM Tissue registry with approval from the McGill University research ethics board, and in accordance with the NIH policy on research in human subjects. Normal control lungs were collected in similar fashion at the McGill University Health Centre. Frozen tissues were processed for Western blotting and formalin-fixed tissues were used for immunohistochemistry. Frozen samples were assessed for the quality of RNA and protein; although the latter was of adequate quality, the lung RNA extracted from patients with LAM was degraded and could not be used to assess the expression of urotensin-related mRNAs.

2.2. Immunohistochemistry

Immunohistochemical stainings for UII, UT, URP (Peninsula labs, CA), von Willebrand factor and HMB45 were performed using the avidin-biotin peroxidase method as previously described [21]. The antibodies against UII and UT utilized in this study have been previously described [7,19]. Paraffin sections were dewaxed in toluene for 20 min, rehydrated in alcohol and washed in phosphate buffered saline (PBS). Following this, sections were incubated with PBS containing Triton-X 100 (0.2%) for 30 min. After three washes with PBS, they were washed with H₂O₂ (3%) to block endogenous peroxidase activity and incubated with 10% normal goat serum for 30 min and then with the primary rabbit anti-sera for 18 h at 4 °C. Finally, sections were incubated with biotinylated IgG (1:200) for 45 min and after three washes with PBS sections were stained with an immunoperoxidase technique according to the manufacturer's instructions (Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA) for 45 min at room temperature. The sections were developed using diaminobenzidine and hydrogen peroxide and then counterstained with hematoxylin, dehydrated, cleared and finally glass cover slips were placed on top of the sections. Negative control sections included immunoabsorption of the primary antisera with their respective antigens, and the use of non-immune serum in place of the primary antibody.

2.3. Western blotting

Western blotting was performed as previously described [45]. Briefly, proteins in tissue homogenates (40 µg) or the control cell extract (Cell Signaling Technologies) were electrophoresed by 10% SDS-PAGE and transferred to a nitrocellulose membrane, which was then incubated with rabbit anti-rat/human UT antibody (1:4000), S6 ribosomal protein antibody (1:1000), or phospho-S6 ribosomal protein (Ser235/236) antibody (1:1000) from Cell Signaling Technologies overnight at 4 °C. Immobilized proteins were incubated with enhanced chemiluminescence reagent (Super-Signal West Pico, Thermo Scientific), and exposed to X-ray film. Images of Western blot films were acquired using an Alpha Imager (Alpha Inotech Corp.), and analyzed with Alpha Ease FC software (V. 4.1.0). Band density was obtained using the spot density and autobackground functions.

2.4. Statistical analyses

Direct two group comparisons were carried out using the student's t-Test. All statistical analyses were carried out using SPSS version 11.5. A P value of <0.05 was considered statistically significant. Data are presented as mean ± standard error.

3. Results

3.1. Immunohistochemistry

In the normal human lung, immunoreactivity for UII was seen in the airway epithelium and glands, and in the endothelial cells of mainly large pulmonary arteries (Figure 1A–C). URP and UT immunoreactivity was also seen in similar distribution, although the presence of UT immunostaining was less apparent in endothelial cells compared to UII and URP (Figure 1D). There was little to no immunostaining for the three molecules over small-medium sized pulmonary arteries. In comparison, there was abundant expression of all three molecules in the lung nodules of patients with LAM (Figure 2A, C and E). There was little to no immunoreactivity in pulmonary artery smooth muscle (Figure 2). Again, immunoreactivity for UII, URP and UT was evident in endothelial cells of mainly large size pulmonary arteries, with UII and URP being more evident than UT (Figure 2B, D and F). Alveolar pneumocytes also expressed all three molecules. In LAM nodules, co-localization by staining of consecutive sections revealed the presence of the three molecules in the same cellular distribution with varying degree of intensity (Figure 2 A , C and E). Presence of the neoplastic marker HMB45

was confirmed in LAM nodules (Figure 1F). Von Willebrand immunoreactivity demonstrated the presence of UII, URP and UT in endothelial cells of pulmonary arteries (data not shown). Although some pulmonary arteries of patients with LAM appeared to have thickened intima and/or media we did not observe a difference in the intensity of the three molecules over endothelial cells of pulmonary arteries of control subjects compared to those of patients with LAM. We occasionally observed UT immunoreactivity over endothelial cells of pulmonary veins and lymphatic vessels in the lungs of patients with LAM. We also noted UT immunoreactivity over myointimal cells of large, but not small, pulmonary arteries. All three molecules were seen in alveolar macrophages. Negative control experiments showed no immunostaining confirming the specificity of the observed signals (Figure 1B).

3.2. Western blotting

In order to quantify the amount of UTR protein expression in the lungs of patients with LAM compared to normal control lungs we performed Western blotting using the same antiserum used for immunohistochemistry. Western blotting revealed the presence of abundant UTR protein in the lungs of patients with LAM (3.34 ± 1.07 AU) compared to negligible amount in the normal (0.0012 ± 0.0007) human lungs (Figure 3A). Indeed, statistical analyses of these data revealed a significant increase in UTR protein expression in the lungs of patients with LAM compared to control subjects ($P < 0.0001$). Consistent with extensive disease in LAM patients undergoing transplantation, two out of three patients exhibited increased phosphorylation of S6, a marker of mTOR activity, when compared to normal controls (1.5 ± 0.3 vs. 0.4 ± 0.05 mean pS6:S6 levels $\pm$ SEM, $p < 0.05$). Regardless of S6 phosphorylation, UT protein levels were elevated in all nine samples from the three patients with LAM when compared to normal control patients. In one patient (LAM-1), S6 phosphorylation was reduced, perhaps reflecting low total protein levels in the sample, or attenuated mTOR activity despite elevated UT levels. Consistent with the former, β -actin levels were also lower in the sample from patient LAM-1. It is also possible that the patient was undergoing treatment with rapamycin, a potent inhibitor of mTOR, at the time of transplantation.

4. Discussion

The present report details the cellular localization of UII and URP, and their receptor UT in the lungs of normal subjects or patients with LAM. Indeed, protein expression of the three molecules was localized to the airway epithelium and glands, and to the endothelium of pulmonary arteries. UT immunoreactivity was also seen over smooth muscle cells of the large size pulmonary arteries. In the lungs of patients with LAM, these three molecules were also expressed in LAM nodules. Interestingly, using Western blotting, we observed little expression of UT protein in the lungs of normal control subjects compared to a significant increase in the lungs of patients with LAM. Previous binding studies demonstrated the presence of low or little UT bindings on the pulmonary vasculature [36] and [37]. In light of the high circulating plasma levels of UII (nanomolar to picomolar) and the pseudo-irreversible nature of UII and URP binding to the UT [15], this makes any significant increase in UT protein expression of great biological significance. Therefore, the present findings points to a potentially significant role for the urotensin II system in the pathogenesis of LAM.

In the present study we demonstrated the presence of UII, URP, UT and HMB45 in LAM nodules. HMB45 positive, or LAM, cells are known to express neural crest and smooth muscle cell markers. Others and we have previously demonstrated the presence of UII in neoplastic cells [35], [41], [47] and [52]. Indeed, UII has been shown to induce growth of cancer cells [53]. Moreover, UII is known to induce proliferation and migration of vascular smooth muscle cells [12], [46] and [56]. Therefore the present demonstration of the three urotensin molecules in the same LAM cells point to either an endocrine and/or paracrine role for UII and URP in

the growth of LAM cells. Indeed, we have recently reported that the use of a selective UT blocker significantly reduced myointimal cell migration and proliferation in carotid arteries following balloon angioplasty opening the door for the possible use of this or similar UT receptor antagonists in LAM [44].

The present demonstration of UII and URP immunoreactivity in endothelial cells of pulmonary arteries is in agreement with other reports describing the pulmonary expression of these two peptides, and their effects on the pulmonary vasculature [1], [9], [37] and [51]. Indeed, UII has been shown to have both vasoconstrictive and vasodilatory properties in the pulmonary vasculature, which are mediated by direct effects on smooth muscle tone and the release of NO and PGI₂ from the endothelium, respectively [26], [36] and [50]. Consistent with an equivocal role for UII in the development of pulmonary hypertension [12], the presence of UII, UT, and URP protein in the thickened small and medium sized pulmonary arteries seen in LAM specimens did not differ from that seen in normal subjects. In fact, pulmonary hypertension is a rare clinical feature of LAM, is thought to be secondary to chronic hypoxemia, and is usually detected in patients with end-stage lung disease [54]. Moreover, we had previously, examined the lungs of 68 patients with different causes of pulmonary hypertension, however we failed to see a trend toward an increase or decrease in UII immunoreactivity (unpublished data). Therefore, the role of the urotensin II system in the pathogenesis of PH remains to be better elucidated using proper pharmacologic or genetic approaches.

Increased UII, UT, and URP expression was primarily observed in HMB45-positive LAM nodules. These lesions are known to exhibit high proliferative capacity in part due to somatic inactivation of the tumor suppressor protein tuberlin [20]. This leads to excessive mTOR activity, cell growth, proliferation, and survival. In agreement, immunodetection of elevated UT protein levels accompanied elevated mTOR activity as detected by phosphorylation of S6. Of note, one patient exhibited reduced phosphorylation of S6, but elevated UT expression in LAM nodules. Others have identified mTOR-independent molecular changes associated with inactivation of tuberlin [8] and [30]. A mechanistic link between urotensin and the survival of tuberlin-deficient cells is the subject of ongoing studies. The UT may also be a therapeutic target in other neurocutaneous syndromes associated with loss of mTOR suppression, such as tuberous sclerosis complex, neurofibromatosis, and Cowden's disease [27].

In summary, this is the first report describing the cellular distribution of the urotensin II system in the normal and diseased human lung. The significantly elevated expression of the UT receptor in the lungs of LAMs suggests a potential role for this important mitogenic peptide in the pathogenesis of this debilitating disease.

Acknowledgments

Dr. Adel Giaid is supported by the Canadian Institute of Health Research and the Heart and Stroke Foundation of Quebec. Dr. Arnold Kristof is supported by NIH R0-CA125436. We acknowledge use of tissues procured by the National Disease Research Interchange (NDRI) with support from NIH grant no. 2U42RR006042-19.

References

1. Ames RS, Sarau HM, Chambers JK, Willette RN, Aiyar NV, Romanic AM, et al. Human urotensin-II is a potent vasoconstrictor and agonist for the orphan receptor GPR14. *Nature* 1999;401:282–286. [PubMed: 10499587]
2. Behm DJ, Ao Z, Camarda V, Aiyar NV, Johns DG, Douglas SA. Inhibitory effects of putative peptidic urotensin-II receptor antagonists on urotensin-II-induced contraction of cat isolated respiratory smooth muscle. *Eur J Pharmacol* 2005;516:276–281. [PubMed: 15975570]

3. Bern HA, Pearson D, Larson BA, Nishioka RS. Neurohormones from fish tails: the caudal neurosecretory system. I. "Urophysiology" and the caudal neurosecretory system of fishes. *Recent Prog Horm Res* 1985;41:533-2.
4. Bottrill FE, Douglas SA, Hiley CR, White R. Human urotensin-II is an endothelium-dependent vasodilator in rat small arteries. *Br J Pharmacol* 2000;130:1865-1870. [PubMed: 10952676]
5. Bousette N, Hu F, Ohlstein EH, Dhanak D, Douglas SA, Giaid A. Urotensin-II blockade with SB-611812 attenuates cardiac dysfunction in a rat model of coronary artery ligation. *J Mol Cell Cardiol* 2006;41:285-295. [PubMed: 16797584]
6. Bousette N, Patel L, Douglas SA, Ohlstein EH, Giaid A. Increased expression of urotensin II and its cognate receptor GPR14 in atherosclerotic lesions of the human aorta. *Atherosclerosis* 2004;176:117-123. [PubMed: 15306183]
7. Bousette N, Pottinger J, Ramli W, Ohlstein EH, Dhanak D, Douglas SA, et al. Urotensin- II receptor blockade with SB-611812 attenuates cardiac remodeling in experimental ischemic heart disease. *Peptides* 2006;27:2919-2926. [PubMed: 16919371]
8. Brugarolas JB, Vazquez F, Reddy A, Sellers WR, Kaelin WG Jr. TSC2 regulates VEGF through mTOR-dependent and -independent pathways. *Cancer Cell* 2003;4:147-158. [PubMed: 12957289]
9. Chatenet D, Dubessy C, Leprince J, Boularan C, Carlier L, Ségalas-Milazzo I, et al. Structure-activity relationships and structural conformation of a novel urotensin II-related peptide. *Peptides* 2004;25:1819-1830. [PubMed: 15476952]
10. Dai HY, Ge ZM, Li YH. Effect of urotensin II on proliferative potential and phosphorylation of extracellular signal-regulated kinase 1/2 of adventitial fibroblasts from spontaneously hypertensive rat. *Zhongguo Yi Xue Ke Xue Yuan Xue Bao* 2006;28:776-780. [PubMed: 17260465]
11. Deuchar GA, Morecroft I, Dempsey Y, Herold N, Nilsen M, Hicks MN, et al. The in vivo effects of human urotensin II in the rabbit and rat pulmonary circulation: effects of experimental pulmonary hypertension. *Eur J Pharmacol* 2006;537:135-142. [PubMed: 16631735]
12. Djordjevic T, BelAiba RS, Bonello S, Pfeilschifter J, Hess J, Görlach A. Human urotensin II is a novel activator of NADPH oxidase in human pulmonary artery smooth muscle cells. *Arterioscler Thromb Vasc Biol* 2005;25:519-525. [PubMed: 15618545]
13. Djordjevic T. Görlach: Urotensin-II in pulmonary vascular remodeling. *Thromb Haemost* 2007;98:952-962. [PubMed: 18000598]
14. Douglas SA. Human urotensin-II as a novel cardiovascular target: 'heart' of the matter or simply a fishy 'tail'? *Curr Opin Pharmacol* 2003;3:159-167. [PubMed: 12681238]
15. Douglas SA, Behm DJ, Aiyar NV, Naselsky D, Disa J, Brooks DP, et al. Nonpeptidic urotensin-II receptor antagonists I: in vitro pharmacological characterization of SB-706375. *Br J Pharmacol* 2005;145:620-635. [PubMed: 15852036]
16. Douglas SA, Dhanak D, Johns DG. From 'gills to pills': urotensin-II as a regulator of mammalian cardiorenal function. *Trends Pharmacol Sci* 2004;25:76-85. [PubMed: 15102493]
17. Douglas SA, Ohlstein EH. Human urotensin-II, the most potent mammalian vasoconstrictor identified to date, as a therapeutic target for the management of cardiovascular disease. *Trends Cardiovasc Med* 2000;10:229-237. [PubMed: 11282300]
18. Douglas SA, Sulpizio AC, Piercy V, Sarau HM, Ames RS, Aiyar NV, et al. Differential vasoconstrictor activity of human urotensin-II in vascular tissue isolated from the rat, mouse, dog, pig, marmoset and cynomolgus monkey. *Br J Pharmacol* 2000;131:1262-1274. [PubMed: 11090097]
19. Douglas SA, Tayara L, Ohlstein EH, Halawa N, Giaid A. Congestive heart failure and expression of myocardial urotensin II. *Lancet* 2002;359:1990-1997. [PubMed: 12076554]
20. Finlay G. The LAM cell: what is it, where does it come from, and why does it grow? *Am J Physiol Lung Cell Mol Physiol* 2004;286:L690-L693. [PubMed: 15003933]
21. Giaid A, Yanagisawa M, Langleben D, Michel RP, Levy R, Shennib H, et al. Expression of endothelin-1 in lungs of patients with pulmonary hypertension. *N Eng J Med* 1993;328:1732-1739.
22. Gibson A. Complex effects of Gillichthys urotensin II on rat aortic strips. *Br J Pharmacol* 1987;91:205-212. [PubMed: 2885055]
23. Gray GA, Jones MR, Sharif I. Human urotensin II increases coronary perfusion pressure in the isolated rat heart: potentiation by nitric oxide synthase and cyclooxygenase inhibition. *Life Sci* 2001;69:175-180. [PubMed: 11441907]

24. Hay DW, Luttmann MA, Douglas SA. Human urotensin-II is a potent spasmogen of primate airway smooth muscle. *Br J Pharmacol* 2000;131:10–12. [PubMed: 10960062]
25. He YH, Hong JM, Guo HS, Wei JR, Chen H, Zuo HH, et al. Effects of urotensin II on cultured cardiac fibroblast proliferation and collagen type I mRNA expression. *Di Yi Jun Yi Da Xue Xue Bao* 2004;24:505–508. [PubMed: 15151818]
26. Hillier C, Berry C, Petrie MC, O'Dwyer PJ, Hamilton C, Brown A, et al. Effects of urotensin II in human arteries and veins of varying caliber. *Circulation* 2001;103:1378–1381. [PubMed: 11245639]
27. Inoki K, Corradetti MN, Guan KL. Dysregulation of the TSC-mTOR pathway in human disease. *Nat Genet* 2005;37:19–24. [PubMed: 15624019]
28. Jegou S, Cartier D, Dubessy C, Gonzalez BJ, Chatenet D, Tostivint H, et al. Localization of the urotensin II receptor in the rat central nervous system. *J Comp Neurol* 2006;49:21–36. [PubMed: 16432902]
29. Karbowniczek M, Astrinidis A, Balsara BR, Testa JR, Lium JH, Colby TV, McCormack FX, Henske EP. Recurrent lymphangiomatosis after transplantation: genetic analyses reveal a metastatic mechanism. *Am J Respir Crit Care Med* 2003;167:976–982. [PubMed: 12411287]
30. Karbowniczek M, Cash T, Cheung M, Robertson GP, Astrinidis A, Henske. Regulation of B-Raf kinase activity by tuberin and Rheb is mammalian target of rapamycin (mTOR)-independent. *J Biol Chem* 2004;279:29930–29937. [PubMed: 15150271]
31. Katano Y, Ishihata A, Aita T, Ogaki T, Horie T. Vasodilator effect of urotensin II, one of the most potent vasoconstricting factors on rat coronary arteries. *Eur J Pharmacol* 2000;402:R5–R7. [PubMed: 10940376]
32. Lin L, Ding WH, Jiang W, Zhang YG, Qi YF, Yuan WJ, et al. Urotensin-II activates L-arginine/nitric oxide pathway in isolated rat aortic adventitia. *Peptides* 2004;25:1977–1984. [PubMed: 15501530]
33. Liu Q, Pong SS, Zeng Z, Zhang Q, Howard AD, Williams DL Jr, et al. Identification of urotensin II as the endogenous ligand for the orphan G-protein-coupled receptor GPR14. *Biochem Biophys Res Commun* 1999;266:174–178. [PubMed: 10581185]
34. Liu JQ, Zelko IN, Erbynn EM, Sham JS, Folz RJ. Hypoxic pulmonary hypertension: role of superoxide and NADPH oxidase (gp91phox). *Am J Physiol Lung Cell Mol Physiol* 2006;290:L2–L10. [PubMed: 16085672]
35. Liu GQ, Zeng ZP, Li HZ, Fan XR, Liu DM, Tong AL, et al. Expression of urotensin II mRNA in normal adrenal medulla and pheochromocytoma tissues. *Zhonghua Yi Xue Za Zhi* 2003;83:1225–1228. [PubMed: 12930635]
36. MacLean MR, Alexander D, Stirrat A, Gallagher M, Douglas SA, Ohlstein EH, et al. Contractile responses to human urotensin-II in rat and human pulmonary arteries: effect of endothelial factors and chronic hypoxia in the rat. *Br J Pharmacol* 2000;130:201–204. [PubMed: 10807654]
37. Maguire JJ, Kuc RE, Davenport AP. Orphan-receptor ligand human urotensin II: receptor localization in human tissues and comparison of vasoconstrictor responses with endothelin-1. *Br J Pharmacol* 2000;131:441–446. [PubMed: 11015293]
38. Matsushita M, Shichiri M, Imai T, Iwashina M, Tanaka H, Takasu N, et al. Co-expression of urotensin II and its receptor (GPR14) in human cardiovascular and renal tissues. *J Hypertens* 2001;19:2185–2190. [PubMed: 11725162]
39. Matsushita M, Shichiri M, Fukai N, Ozawa N, Yoshimoto T, Takasu N, et al. Urotensin II is an autocrine/paracrine growth factor for the porcine renal epithelial cell line, LLCPK1. *Endocrinology* 2003;144:1825–1831. [PubMed: 12697688]
40. Mori M, Sugo T, Abe M, Shimomura Y, Kurihara M, Kitada C, et al. Urotensin II is the endogenous ligand of a G-protein-coupled orphan receptor, SENR (GPR14). *Biochem Biophys Res Commun* 1999;265:123–129. [PubMed: 10548501]
41. Morimoto R, Satoh F, Murakami O, Totsune K, Arai Y, Suzuki T, et al. Immunolocalization of urotensin II and its receptor in human adrenal tumors and attached non-neoplastic adrenal tissues. *Peptides* 2008;29:873–880. [PubMed: 17686550]
42. Nothacker HP, Wang Z, McNeill AM, Saito Y, Merten S, O'Dowd B, et al. Identification of the natural ligand of an orphan G-proteincoupled receptor involved in the regulation of vasoconstriction. *Nat Cell Biol* 1999;1:383–385. [PubMed: 10559967]

43. Pearson D, Shively JE, Clark BR, Geschwind II, Barkley M, Nishioka RS, et al. Urotensin II: a somatostatin-like peptide in the caudal neurosecretory system of fishes. *Proc Natl Acad Sci USA* 1980;77:5021–5024. [PubMed: 6107911]
44. Rakowski E, Hassan G, Ohlstein EH, Douglas SA, Giaid A. A role for urotensin II in restenosis following balloon angioplasty: use of a selective UT receptor blocker. *J Mol Cell Cardiol* 2005;39:785–791. [PubMed: 16171813]
45. Saito T, Hu F, Tayara L, Fahas L, Shennib H, Giaid A. Inhibition of NO synthase II prevents cardiac dysfunction in myocardial infarction and congestive heart failure. *Am J Physiol: Heart and Circulatory Physiology* 2002;283:H339–H345.
46. Sauzeau V, Le Mellionec E, Bertoglio J, Scalbert E, Pacaud P, Loirand G. Human urotensin II-induced contraction and arterial smooth muscle cell proliferation are mediated by RhoA and Rho-kinase. *Circ Res* 2001;88:1102–1104. [PubMed: 11397774]
47. Shenouda A, Douglas SA, Olstein EH, Giaid A. Localization of urotensin-II immunoreactivity in normal human kidneys and renal carcinoma. *Histochem Cytochem* 2002;50:885–890.
48. Shi L, Ding W, Li D, Wang Z, Jiang H, Zhang J, et al. Proliferation and antiapoptotic effects of human urotensin II on human endothelial cells. *Atherosclerosis* 2006;188:260–264. [PubMed: 16343502]
49. Spinazzi R, Albertin G, Nico B, Guidolin D, Di Liddo R, Rossi GP, et al. Urotensin-II and its receptor (UT-R) are expressed in rat brain endothelial cells, and urotensin-II via UT-R stimulates angiogenesis in vivo and in vitro. *Int J Mol Med* 2006;18:1107–1112. [PubMed: 17089015]
50. Stirrat A, Gallagher M, Douglas SA, Ohlstein EH, Berry C, Kirk A, et al. Potent vasodilator responses to human urotensin-II in human pulmonary and abdominal resistance arteries. *Am J Physiol Heart Circ Physiol* 2001;280:H925–H928. [PubMed: 11158995]
51. Sugo T, Murakami Y, Shimomura Y, Harada M, Abe M, Ishibashi Y, et al. Identification of urotensin II-related peptide as the urotensin II-immunoreactive molecule in the rat brain. *Biochem Biophys Res Commun* 2003;310:860–868. [PubMed: 14550283]
52. Takahashi K, Totsune K, Kitamuro T, Sone M, Murakami O, Shibahara S. Three vasoactive peptides, endothelin-1, adrenomedullin and urotensin-II, in human tumour cell lines of different origin: expression and effects on proliferation. *Clin Sci (Lond)* 2002;103:S35–S38.
53. Takahashi K, Totsune K, Murakami O, Arihara Z, Noshiro T, Hayashi Y, et al. Expression of urotensin II and its receptor in adrenal tumors and stimulation of proliferation of cultured tumor cells by urotensin II. *Peptides* 2003;24:301–306. [PubMed: 12668216]
54. Taveira-DaSilva AM, Hathaway OM, Sachdev V, Shizukuda Y, Birdsall CW, Moss J. Pulmonary artery pressure in lymphangioleiomyomatosis: an echocardiographic study. *Chest* 2007;132:1573–1578. [PubMed: 17890459]
55. Wang H, Mehta JL, Chen K, Zhang X, Li D. Human urotensin II modulates collagen synthesis and the expression of MMP-1 in human endothelial cells. *J Cardiovasc Pharmacol* 2004;44:577–581. [PubMed: 15505495]
56. Watanabe T, Pakala R, Katagiri T, Benedict CR. Synergistic effect of urotensin II with serotonin on vascular smooth muscle cell proliferation. *J Hypertens* 2001;19:2191–2196. [PubMed: 11725163]
57. Zeng ZP, Liu GQ, Li HZ, Fan XR, Liu DM, Tong AL, et al. The effects of urotensin-II on proliferation of pheochromocytoma cells and mRNA expression of urotensin-II and its receptor in pheochromocytoma tissues. *Ann N Y Acad Sci* 2006;1073:284–289. [PubMed: 17102097]
58. Zhang Y, Li J, Cao J, Chen J, Yang J, Zhang Z, et al. Effect of chronic hypoxia on contents of urotensin II and its functional receptors in rat myocardium. *Heart Vessels* 2002;16:64–68. [PubMed: 11833844]

Fig. 1.
Immunohistochemical localization of UII and UT in lungs of normal control subjects, and MMB45 in nodule of LAM patient.
Panels A–C demonstrates UII immunoreactivity in the airway epithelium (A and B; arrow heads), vascular endothelium of large-sized pulmonary artery (A; arrow) and bronchial glands (C; arrows). Panel D shows UT immunoreactivity in bronchial glands (arrows). Panel E represents a negative control section immunostained with the non-immune serum. Panel F shows the localization of MMB45 immunoreactivity in LAM nodule (arrow).

U11

URP

UT

Fig. 2.

Immunohistochemical localization of U11, UT and URP in lungs of patients with LAM. U11 immunoreactivity is evident in LAM nodule (A) and endothelium of pulmonary artery (B arrow). URP (C) and UT (E) immunoreactivities shown in the same LAM nodule and in the endothelium (arrow) of pulmonary arteries (D and F, respectively).

A **B** **Fig. 3.**

Western blot of UTR (A) and S6 ribosomal protein and phospho-S6 ribosomal protein expression (B) in p70 S6 kinase control cell extract (Ctrl), the lungs of 3 patients with LAM (1–10), and in normal human lungs (1–3 Ctrl).